The Neurobiology of Autism

Second Edition

Edited by
Margaret L. Bauman, M.D.,
and Thomas L. Kemper, M.D.

VISIT…

THE NEUROBIOLOGY OF AUTISM

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SECOND EDITION

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PREFACE

Since the first edition of this book was published in 1994, clinical and basic science research in autism has expanded substantially, due in large part to parent advocacy, the availability of greater financial support for young investigators, increased federal funding for senior scientists, and the development of national and international collaborations. As a result, significant progress has been made in our clinical and neurobiologic understanding of this disorder. Despite these advances, however, we still have much to learn. The etiology of autism remains largely unknown, and there are still no metabolic, genetic, or radiographic markers to aid in the diagnosis or predict clinical severity. It has become increasingly apparent that, in terms of management and intervention, strategies that are effective for one child are not necessarily effective for another. Thus there is a spectrum of symptoms, clinical presentations, and severities, which we do not yet fully understand.

This book provides a sample of the current active research in the field of autism. It is our hope that having this information readily available in a single resource will assist and stimulate present and future investigators to consider a broad range of novel research questions that will further expand and extend what is now known about this disorder. We hope that strategies will ultimately be defined to aid in early diagnosis and to develop more focused and effective treatment.

We extend our sincere thanks to our families, who have allowed each of us the time to conduct our time-consuming research and assemble this book. We also thank our autistic patients, who have inspired us, and we extend our gratitude to their families, teachers, therapists, and physicians, who spend long hours educating themselves and who daily provide the intensive services from which these children, adolescents, and adults best benefit. Many autistic individuals have the potential to succeed, to make significant progress, and to live successful and meaningful lives. We dedicate this book to them, with our personal commitment to continue the search for answers.

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1

The Epidemiology of Pervasive Developmental Disorders

Eric Fombonne, M.D., FRCPsych

Epidemiologic surveys of autism started in the mid-1960s in England (Lotter, 1966) and since then have been conducted in many countries. All epidemiologic surveys have focused on a categorical-diagnostic approach to autism that over time has relied on different sets of criteria; however, all surveys relied on a definition of autism that comprised severe impairments in communication and language, social interaction, and play and behavior. With the exception of recent studies, other pervasive developmental disorders (PDDs) falling short of diagnostic criteria for autism (PDD-NOS [not otherwise specified], Asperger syndrome) were generally not included in the definition used in the earlier surveys, although several epidemiologic investigations yielded useful information on the rates of these particular types of PDD. These data are summarized separately. This chapter reviews the methodologic features and substantive results of published epidemiologic surveys, then addresses the following questions: (1) What is the range of prevalence estimates for autism and related disorders? (2) What are the correlates of autism? (3) Is the incidence of autism increasing? (4) How many children have a PDD in the United States today?

Selection of Studies

The studies were identified through systematic searches of scientific literature databases (Medline, PsycINFO) and from prior reviews (Wing, 1993; Fombonne, 1999). Only studies published in the English language were included in this review. Overall, 37 studies published between 1966 and early 2003 were selected that surveyed PDDs in clearly demarcated, nonoverlapping samples.

Study Designs

The surveys were conducted in 14 countries, and half of the results were published during the past decade. Details on the precise sociodemographic compo-

sition and economic activities of the area surveyed in each study were generally lacking; however, most studies were conducted in predominantly urban or mixed areas. The age range of the population included in the surveys is from 3 years to early adult life, with an overall median age of 8.0 years across the 34 studies. Similarly, there is huge variation in the size of the population surveyed (range: 826–4,600,000), with a median population size of 65,300 subjects and about half of the studies relying on targeted populations ranging in size from 16,000 to 152,000.

Most investigations relied on a two-stage or multiple-stage approach to identify cases in underlying populations. The first screening stage of these studies often consisted of sending letters or brief screening scales requesting school and health professionals to identify possible cases of autism. Each investigation varied in several key aspects of this screening stage. First, the coverage of the population varied enormously from one study to another. In some (i.e., studies 3, 17, 20, 24, and 34), only cases already known from educational or medical authorities could be identified, whereas in other surveys, an extensive coverage of the entire population was achieved, including children attending normal schools (studies 1 and 25) or children undergoing systematic developmental checks (studies 13, 19, 22, and 32). In addition, the surveyed areas varied in terms of service development as a function of the specific educational or health care systems of each country and of the year of investigation. Second, the type of information sent out to professionals invited to identify children varied from simple letters including a few clinical descriptors of autism-related symptoms or diagnostic checklists rephrased in nontechnical terms to more systematic screening based on questionnaires or rating scales of known reliability and validity. Third, participation rates in the first screening stage are another source of variation in the screening efficiency of surveys. However, refusal rates were low (median: 10% in 11 studies).

The sensitivity of the screening methodology is difficult to gauge in autism surveys. The usual epidemiologic approach, which consists of sampling at random screened negative subjects to estimate the proportion of false negatives, was not used in these surveys for the obvious reason that, due to the very low frequency of the disorder, it would be both imprecise and very costly to undertake such estimations. Prevalence estimates must therefore be seen as underestimates of "true" prevalence rates, because cases are being missed due to either lack of cooperation or imperfect sensitivity of the screening procedure. The magnitude of this underestimation is unknown in each survey.

Similar considerations about the methodologic variability across studies apply to the intensive assessment phases. Participation rates were not always available but were generally high (range: 76.1%–98.6%). The source of information used to

determine identification of cases usually involved a combination of informants and data sources, with a direct assessment of the person with autism in 19 studies. The assessments were conducted with various diagnostic instruments, ranging from a classical clinical examination to the use of batteries of standardized measures. The precise diagnostic criteria retained to define caseness varied according to the study and, to a large extent, reflected historical changes in classification systems (Table 1.1). However, most surveys relied on the clinical judgment of experts to arrive at the final case groupings, and it is thus difficult to assess the impact of a specific diagnostic criterion or algorithm on the estimate of prevalence.

Estimates of the Prevalence of Autistic Disorder

In 34 of the 37 studies that provided data on autistic disorder, prevalence estimates ranged from 0.7/10,000 to 72.6/10,000 (Table 1.1). Prevalence rates were negatively correlated with sample size (Spearman $r = -0.71$; $p < 0.01$); small-scale studies tended to report higher prevalence rates. The median prevalence rate for 18 surveys published in the period 1966–1992 was 4.6/10,000, and the median rate for the 16 surveys published in the period 1993–2003 was 12.7/10,000. Indeed, the correlation between prevalence rate and year of publication reached statistical significance (Spearman $r = 0.65$; $p < 0.01$); the results of the 18 surveys with prevalence rates greater than 7/10,000 were all published since 1987. To derive a best estimate of the current prevalence of autism, this review restricted analysis to the 26 most recent surveys, published since 1987. Five studies with a very small target population (>10,000) were excluded, as their estimates were very imprecise. For the 21 remaining studies, the estimates ranged from 2.5/10,000 to 30.8/10,000 (average 95% CI width: 6.2), with an average rate of 11.0/10,000 and a median rate of 10.0/10,000. Similar values were obtained when slightly different rules and time cutpoints were used. For further calculations, 10/10,000 was adopted as the working rate for the prevalence of autism.

Correlates of Autism

GENDER, INTELLECTUAL LEVEL, AND SOCIAL CLASS

The male:female sex ratio varies from 1.33:1 (study 7) to 16.0:1 (study 4), with a mean of 4.4:1. The male excess is more pronounced when autism is not associated with mental retardation (M:F ratio: 5.8:1, 12 studies). The median proportion of subjects without intellectual impairment is 30 percent (range: 0%–60%). The corresponding figures are 30 percent (range: 6.6%–100%) for mild to moderate

TABLE 1.1. Prevalence Surveys of Autistic Disorder

Number	*Study*	*Location*	*Size of Target Population*	*Number of Subjects with Autism*	*Diagnostic Criteria*	*Percentage with Normal IQ*	*Ratio (M:F)*	*Gender Prevalence Rate/ 10,000*	*95% CI*
1	Lotter (1966)	Middlesex, United Kingdom	78,000	32	Rating scale	15.6	2.6 (23/9)	4.1	2.7; 5.5
2	Brask (1970)	Aarhus County, Denmark	46,500	20	Clinical	—	1.4 (12/7)	4.3	2.4; 6.2
3	Treffert (1970)	Wisconsin, United States	899,750	69	Kanner	—	3.06 (52/17)	0.7	0.6; 0.9
4	Wing et al. (1976)	Camberwell, United Kingdom	25,000	17[a]	Twenty-four-item rating scale of Lotter	30	16 (16/1)	4.8[b]	2.1; 7.5
5	Hoshino et al. (1982)	Fukushima-Ken, Japan	609,848	142	Kanner	—	9.9 (129/13)	2.33	1.9; 2.7
6	Bohman et al. (1983)	Västerbotten County, Sweden	69,000	39	Rutter	20.5	1.6 (24/15)	5.6	3.9; 7.4
7	McCarthy et al. (1984)	East Ireland	65,000	28	Kanner	—	1.33 (16/12)	4.3	2.7; 5.9
8	Steinhausen et al. (1986)	West Berlin, Germany	279,616	52	Rutter	55.8	2.25 (36/16)	1.9	1.4; 2.4
9	Burd et al. (1987)	North Dakota, United States	180,986	59	DSM-III	—	2.7 (43/16)	3.26	2.4; 4.1

10	Matsuishi et al. (1987)	Kurume City, Japan	32,834	51	DSM-III	—	4.7 (42/9)	15.5	11.3; 19.8
11	Tanoue et al. (1988)	Southern Ibaraki, Japan	95,394	132	DSM-III	—	4.07 (106/26)	13.8	11.5; 16.2
12	Bryson et al. (1988)	Part of Nova Scotia, Canada	20,800	21	New RDC	23.8	2.5 (15/6)	10.1	5.8; 14.4
13	Sugiyama and Abe (1989)	Nagoya, Japan	12,263	16	DSM-III	—	—	13.0	6.7; 19.4
14	Cialdella and Mamelle (1989)	One department (Rhône) France	135,180	61	DSM-III–like	—	2.3	4.5	3.4; 5.6
15	Ritvo et al. (1989)	Utah, United States	769,620	241	DSM-III	34	3.73 (190/51)	2.47	2.1; 2.8
16	Gillberg et al. (1991)[c]	Southwest Göteburg and Bohuslän County, Sweden	78,106	74	DSM-III-R	18	2.7 (54/20)	9.5	7.3; 11.6
17	Fombonne and du Mazaubrun (1992)	Four regions, 14 departments, France	274,816	154	Clinical ICD-10–like	13.3	2.1 (105/49)	4.0	94.1; 5.7
18	Wignyosumarto et al. (1992)	Yogyakarita, Indonesia (southeast of Jakarta)	5,120	6	CARS	0	2.0 (4/2)	11.7	2.3; 21.1
19	Honda et al. (1996)	Yokohama, Japan	8,537	18	ICD-10	50.0	2.6 (13.5)	21.08	11.4; 30.8

(continued)

TABLE 1.1. Continued

Number	*Study*	*Location*	*Size of Target Population*	*Number of Subjects with Autism*	*Diagnostic Criteria*	*Percentage with Normal IQ*	*Ratio (M:F)*	*Gender Prevalence Rate/ 10,000*	*95% CI*
20	Fombonne et al. (1997)	Three departments, France	325,347	174	Clinical ICD-10–like	12.1	1.81 (112/62)	5.35	4.6; 6.1
21	Webb et al. (1997)	South Glamorgan, United Kingdom	73,301	53	DSM-III-R	—	6.57 (46/7)	7.2	5.3; 9.3
22	Arvidsson et al. (1997)	Mölnlycke, Sweden (west coast)	1,941	9	ICD-10	22.2	3.5 (7/2)	46.4	16.1; 76.6
23	Sponheim and Skjeldal (1998)	Akershus County, Norway	65,688	34	ICD-10	47.1[d]	2.09 (23/11)	5.2	3.4; 6.9
24	Taylor et al. (1999)	North Thames, United Kingdom	≈490,000	427	ICD-10	—	—	8.7	7.9; 9.5
25	Kadesjö et al. (1999)	Karlstad, Sweden (central)	826	6	DSM-III-R/ ICD-10; Gillberg (Asperger syndrome)	50	5.0 (5/1)	72.6	14.7; 130.6
26	Baird et al. (2000)	Southeast Thames, United Kingdom	16,235	50	ICD-10	60	15.7 (47/3)	30.8	22.9; 40.6

27	Powell et al. (2000)	West Midlands, United Kingdom	25,377	62	Clinical/ ICD-10; DSM-IV	—	—	7.8	5.8; 10.5
28	Kielinen et al. (2000)	Oulu and Lapland, Finland (north)	152,732	187	ICD-8/ICD-9/ ICD-10	49.8	4.12 (156/50)	12.2	10.5; 14.0
29	Bertrand et al. (2001)	Brick Township, New Jersey United States	8,896	36	DSM-IV	36.7	2.2 (25/11)	40.5	28.0; 56.0
30	Fombonne et al. (2001a)	England and Pays de Galles, United Kingdom	10,438	27	DSM-IV/ ICD-10	55.5	8.0 (24/3)	26.1	16.2; 36.0
31	Magnússon and	Iceland (entire island) Saemundsen (2001)	43,153	57	Mostly ICD-10	15.8	4.2	13.2 (46/11)	9.8; 16.6
32	Chakrabarti and Fombonne (2001)	Staffordshire, United Kingdom	15,500	26	ICD10/ DSM-IV	29.2	3.3 (20/6)	16.8	10.3; 23.2
33	Davidovitch et al. (2001)	Haifa, Israel	26,160	26	DSM-III-R/ DSM-IV	—	4.2	10.0	6.6; 14.4
34	Croen et al. (2002)	California, United States	4,590,333	5,038	CDER "Full syndrome"	62.8	4.47 (4116/921)	11.0	10.7; 11.3

Note: —, no data available.

[a] This number corresponds to the sample described in Wing and Gould (1979).

[b] This rate corresponds to the first published paper on this survey and is based on 12 subjects among children aged 5–14 years.

[c] For the Göteborg surveys by Gillberg et al. (Gillberg, 1984; Steffenburg and Gillberg, 1986; Gillberg et al., 1991), a detailed examination showed that there was overlap between the samples included in the three surveys; consequently, only the last survey has been included in this table.

[d] In this study, mild mental retardation was combined with normal IQ, whereas moderate and severe mental retardation were grouped together.

intellectual impairment and 40 percent (range: 0%–81.3%) for severe to profound mental retardation. In four earlier surveys (studies 1, 2, 3, and 5), an association has been reported between autism and higher educational or social class parental status. However, in eight studies conducted after 1980, social class was no longer associated with autism. The earlier findings probably reflected a bias due to differential access to services by social class, as shown in other large clinical samples (Schopler et al., 1979; Wing, 1980). More recently, however, Croen et al. (2002) reported an association between autism and higher maternal educational status in a large survey.

MEDICAL DISORDERS

Rates of medical conditions associated with autism have been noted in 15 surveys, and details are reported elsewhere (Fombonne, 2003b). To summarize, the overall proportion of cases of autism that could be causally attributed to known medical disorders remains low. From the 14 surveys in which rates were available for one of seven clear-cut medical disorders potentially causally associated with autism (cerebral palsy, fragile X syndrome, Tourette syndrome, phenylketonuria [PKU], neurofibromatosis, congenital rubella, and Down syndrome), it is estimated that the attributable proportion of cases of autism would be near 6 percent for any medical disorder (excluding epilepsy and sensory impairments).

Rates of epilepsy are high among autism samples. The proportion of autistic individuals with epilepsy also tends to be higher in those studies that include subjects with higher rates of severe mental retardation (as in studies 16, 17, and 20). Age-specific rates for the prevalence of epilepsy were not available. The samples in which high rates of epilepsy were reported tended to have a higher median age, although these rates seemed to apply primarily to school-aged children. Thus, in the light of the increased incidence of seizures during adolescence among subjects with autism (Deykin and MacMahon, 1979; Volkmar and Nelson, 1990), the epidemiologic rates should be regarded as underestimates of the lifetime risk of epilepsy in autism.

Rates of Other Pervasive Developmental Disorders

UNSPECIFIED PERVASIVE DEVELOPMENTAL DISORDERS

Several studies have provided useful information on rates of syndromes similar to autism but falling short of strict diagnostic criteria (Table 1.2). Because the screening procedures and subsequent diagnostic assessments have differed from

one study to another, these groups of disorders are not strictly comparable across studies. Although details are often lacking on their phenomenologic features in the available reports, these disorders would currently be included in the atypical autism and unspecified pervasive developmental disorders (PDD-NOS) diagnostic categories. Twelve of the 34 surveys yielded estimates of the prevalence of these developmental disorders, with eight studies showing higher rates for the broader phenotype than for autism. The ratio of the rate of PDD-NOS to the rate of autism varied from 0.44 to 3.33 (Table 1.2) with a mean value of 1.5, which translates into an average prevalence estimate of 15/10,000. In other words, for every two children assessed with autism in epidemiologic surveys, another three children were found to have severe impairments of a similar nature but falling short of the strict diagnostic criteria for autism. Two other studies (Scott et al., 2002; Yeargin-Allsopp et al., 2003) provided an estimate of the prevalence rate of all PDDs, but no breakdown between autistic disorder and PDD-NOS rates was provided.

Asperger Syndrome and Childhood Disintegrative Disorder

Epidemiologic studies of Asperger syndrome are sparse, reflecting the recency of its inclusion as a separate diagnostic category in both ICD-10 and DSM-IV. Two epidemiologic surveys specifically investigated its prevalence (Ehlers and Gillberg, 1993; Kadesjö et al., 1999). However, only a handful ($N < 5$) of cases were identified in these surveys, with the resulting estimates of 28/10,000 and 48/10,000 being extremely imprecise. One survey of Cardiff schools estimated the prevalence of both high-functioning autism/PDD-NOS and of Asperger syndrome to be 20/10,000 (Webb et al., 2003); unfortunately, no separate estimate was available for Asperger syndrome only. By contrast, other recent autism surveys have consistently identified *smaller* numbers of children with Asperger syndrome than those with autism within the same survey. The ratio of autism to Asperger syndrome rates in each survey was above unity, suggesting that the rate of Asperger syndrome was consistently lower than that for autism. How much lower is difficult to establish from existing data, but a ratio of 4:1 would appear to be an acceptable, albeit conservative, conclusion (Fombonne, 2001a). This translates into a rate for Asperger syndrome which would be one-quarter that of autism or an estimated rate of 2.5/10,000.

Few surveys have provided data on childhood disintegrative disorder (for a review, see Volkmar, 1992). In four studies, prevalence estimates ranged from 11.1 to 64.5 per million and the pooled estimate was 1.7/100,000 (Fombonne, 2002). Childhood disintegrative disorder is thus extremely rare.

TABLE 1.2. Rates of Nonautism Pervasive Developmental Disorders (PDDs)

Number	*Study*	*Rate of Autism*	*Prevalence Rate of Other PDDs*	*Combined Rate of Autism and Other PDDs*	*Prevalence Rate Ratio*[a]	*Case Definition for Other PDDs*
1	Lotter (1966)	4.1	3.3	7.8	0.90	Some behavior similar to autistic children
2	Brask (1970)	4.3	1.9	6.2	0.44	"Other psychoses" or "borderline psychotic"
4	Wing et al. (1976)	4.9	16.3	21.2	3.33	Socially impaired (triad of impairments)
5	Hoshino et al. (1982)	2.33	2.92	5.25	1.25	Autistic mental retardation
9	Burd et al. (1987)	3.26	>7.79[b]	>11.05[b]	2.39	Referred by professionals with "autisticlike" symptoms, not meeting DSM-III criteria for IA, COPDD, or atypical PDD
14	Cialdella and Mamelle (1989)	4.5	4.7	9.2	1.04	Meeting criteria for other forms of "infantile psychosis" than autism, or a broadened definition of DSM-III
17	Fombonne and du Mazaubrun (1992)[c]	4.6	6.6	11.2	1.43	Mixed developmental disorders
20	Fombonne et al. (1997)	5.3	10.94	16.3	2.05	Mixed developmental disorders

26	Baird et al. (2000)	30.8	27.1	57.9	0.9	Other PDDs
27	Powell et al. (2000)	7.8	13.0	20.8	1.7	Other PDDs
29	Bertrand et al. (2001)	40.5	27.0	67.4	0.7	PDD-NOS and Asperger syndrome
32	Chakrabarti and Fombonne (2001)	16.8	36.1	52.9	2.15	PDD-NOS
35	Scott et al. (2002)	—	—	57.0	—	All PDDs
36	Yeargin-Allsopp et al. (2003)	—	—	34.0	—	All PDDs

Note: —, no data available.

[a] Other PDD rate divided by autism rate.

[b] Computed by the author.

[c] These rates are derived from the complete results of the survey of three birth cohorts of French children (Rumeau-Rouquette, 1996).

Prevalence of All Pervasive Developmental Disorders

Taking the aforementioned conservative estimates, the prevalence for all PDDs is at least 27.5/10,000 (i.e., the sum of estimates for autism [10/10,000], PDD-NOS [15/10,000], and Asperger syndrome [2.5/10,000]). This global estimate is derived from a conservative analysis of existing data and is consistent with the results of the large survey conducted by the Centers for Disease Control and Prevention in Atlanta (Yeargin-Allsopp et al., 2003).

Newer Surveys

Three recent epidemiologic surveys have yielded rates about twice as high as the above conservative estimate for PDD prevalence (Table 1.3). The common design features of these epidemiologic inquiries are worth noting. First, the case definition chosen for these investigations was that of a pervasive developmental disorder (PDD), as opposed to a narrower approach focusing on autistic disorder. Investigators were concerned with *any* combination of severe developmental abnormalities occurring in one or more of the three symptomatic domains defining PDD and autism. Second, case-finding techniques employed in these surveys were proactive, relying on multiple and repeated screening phases, both involving different informants at each phase and surveying the same cohorts at different ages, which certainly maximized the sensitivity of case identification. Third, assessments were performed with standardized diagnostic measures (i.e., ADI-R and ADOS) that closely match the more dimensional and broader approach retained for case definition. Finally, these samples comprised young children around the time of their fifth birthday, thereby optimizing the sensitivity of case-finding procedures, as false negatives are less likely around that age. Conducted in different regions and countries by different teams, the convergence of estimates (Table 1.3, right column) is striking. Two further results are worth noting. First, in sharp contrast with the prevalence for combined PDDs, the separate estimates for autistic disorder and PDD-NOS and/or Asperger syndrome vary widely across studies, as if the reliability of the differentiation between autistic disorder and PDD-NOS was mediocre at that young age, despite the use of up-to-date standardized measures. This variability precluded partitioning the overall PDD rate of 60/10,000 into estimates for each diagnostic subtype. Second, the rate of mental retardation was, overall, much lower than in previous surveys of autism. Although this should not be a surprise for children in the PDD-NOS/Asperger syndrome groups, this trend was also noticeable within the samples diagnosed with autistic disorder. To what extent the trend reflects

TABLE 1.3. Recent Epidemiologic Surveys of Pervasive Developmental Disorders (PDDs)

		Autism			*PDD-NOS + Asperger*			*All PDDs*
Study	*Age (years)*	*Rate/ 10,000*	*M:F Ratio*	*Percentage Normal IQ*	*Rate/ 10,000*	*M:F Ratio*	*Percentage Normal IQ*	*Rate/ 10,000*
Bertrand et al. (2001)	3–10	40.5	2.2	37	27.0	3.7	51	67.5
Baird et al. (2000)	7	30.8	15.7	60	27.1	4.5	—	57.9
Chakrabarti and Fombonne (2001)	4–7	16.8	3.3	29	44.5	4.3	94	61.3

Note: —, no data available.

some degree of misclassification between autism and PDD-NOS or a genuine trend over time toward a decreased rate of mental retardation for children with autistic disorder (possibly as a result of earlier diagnosis and intervention) remains to be established.

Time Trends

The debate on the hypothesis of a secular increase in rates of autism has been obscured by a lack of clarity in the measures of disease occurrence used by investigators, or rather, in their interpretation (Fombonne, 2003a). In particular, it is crucial to differentiate prevalence (the proportion of individuals in a population who have a defined disorder at a given point in time) from incidence (the number of new cases occurring in a population over a period of time). Prevalence is useful to estimate needs and plan services; only incidence rates can be used for causal research. Both prevalence and incidence estimates will be inflated when case definition is broadened and case ascertainment is improved. Time trends in rates can therefore be gauged only in investigations that hold these parameters under strict control over time. Although incidence data are required to best examine secular changes in disease occurrence, five approaches to assess this question have been used in the literature.

REFERRAL STATISTICS

Increasing numbers of children referred to specialist services or known to special education registers have been taken as evidence for an increased incidence of autism spectrum disorders. However, trends over time in referred samples are confounded by many factors, such as referral patterns, availability of services, heightened public awareness, decreasing age at diagnosis, and changes over time in diagnostic concepts and practices, to name only a few. Failure to control for these confounding factors is obvious in some recent reports, such as the widely quoted report from California's educational services (California Department of Developmental Services, 1999; Fombonne, 2001b).

COMPARISON OF CROSS-SECTIONAL EPIDEMIOLOGIC SURVEYS

Due to their cross-sectional methodology, most epidemiologic investigations of autism have been concerned with prevalence estimates of autism. As shown earlier, epidemiologic surveys of autism each possess unique design features that could account almost entirely for between-study variations in rates, and time trends in rates of autism are therefore difficult to gauge from published prevalence rates. The significant correlation previously mentioned between prevalence rate and year of publication could merely reflect increased efficiency over time in case-identification methods used in surveys, as well as changes in diagnostic concepts and practices.

The most convincing evidence that method factors could account for most of the variability in published prevalence estimates comes from two sets of surveys conducted in the United Kingdom and the United States (Table 1.4). In each country, four surveys were conducted around the same year and with similar age groups. As there is no reason to expect huge between-area differences in rates, prevalence estimates should be closely comparable within each country. Nevertheless, a sixfold variation in rates is observed for U.K. surveys and a thirteenfold variation in U.S. rates. In each set, high rates derive from surveys in which intensive population-based screening techniques were employed, whereas lower rates were obtained from studies relying on administrative methods for case finding. Because no passage of time was involved, the magnitude of these gradients in rates can be safely attributed to differences in case-definition and case-identification methods across surveys; the replication of the pattern in two countries provides even more confidence in this interpretation. Thus, no inference on secular trends in the incidence of PDDs can be derived from a simple comparison of prevalence rates over time, because studies conducted at different periods are likely to differ even more with respect to their methodology.

TABLE 1.4. Impact of Study Design on Prevalence Estimates

Study	*Location*	*Size*	*Age Group (years)*	*Method*	*PDD Rate/ 10,000*
U.K. Studies					
Chakrabarti and Fombonne (2001)	Staffordshire, United Kingdom	15,500	2.5–6.5	Intense screening and assessment	62.6
Baird et al. (2000)	Southeast Thames, United Kingdom	16,235	7	Early screening and follow-up identification	57.9
Fombonne et al. (2001)	England and Wales	10,438	5–15	National household survey of psychiatric disorders	26.1
Taylor et al. (1999)	North Thames, United Kingdom	490,000	0–16	Administrative records	10.1
U.S. Studies					
Bertrand et al. (2001)	Brick Township, New Jersey, United States	8,896	3–10	Multiple sources of ascertainment	67
Sturmey and James (2001)	Texas, United States	3,564,577	6–18	Educational services	16
CDER (1999)	California, United States	3,215,000	4–9	Educational services	15
Hillman et al. (2000)	Missouri, United States	—	5–9	Educational services	4.8

Note: —, no data available.

The next two approaches are in essence comparable to this one, although specific attempts are made to maintain some design features of the surveys constant.

REPEATED SURVEYS IN DEFINED GEOGRAPHICAL AREAS

Repeated surveys, using the same methodology and conducted in the same geographical area at different points in time, can potentially yield useful information on time trends, provided that methods are kept relatively constant. The Göteborg studies (Gillberg, 1984; Gillberg et al., 1991) provided three prevalence estimates that increased over a short period of time from 4.0/10,000 (1980) to 6.6/10,000 (1984) and 9.5/10,000 (1988) (Gillberg et al., 1991). However, comparisons of these rates are not straightforward, as different age groups were included in each survey. The increased prevalence in the second survey was explained by improved detection among the mentally retarded, and that of the third survey by cases born to parents migrating into the area (Gillberg, 1987). Taken in conjunction with a change in local services and a progressive broadening of the definition of autism over time acknowledged by the authors, these findings do not provide solid evidence for an increased incidence in the rate of autism.

SUCCESSIVE BIRTH COHORTS

In large surveys encompassing a wide age range, higher prevalence rates among the most recent birth cohorts could be interpreted as indicating a secular increase in the incidence of the disorder, provided that alternative explanations can confidently be ruled out. Pooling data from three French surveys that relied on rigorously similar methods, age-specific rates in birth cohorts from 1971 to 1985 showed no upward trend (Fombonne et al., 1997). These results derived from a total target population of 735,000 children, 389 of whom had autism. However, children with autism and substantial mental retardation were mostly reflected in these studies and, as a consequence, any upward trend applying specifically to high-functioning subjects might have gone undetected.

INCIDENCE STUDIES

Only two studies have provided incidence estimates (Powell et al., 2000; Kaye et al., 2001). Both studies showed an upward trend in incidence over short periods of time, but no attempt was made in either investigation to assess changes over the corresponding periods in diagnostic criteria and sensitivity of case-detection methodology.

The available epidemiologic evidence does not support the hypothesis that the incidence of autism has increased, and several other reasons could easily account for an artifactual impression of an increase. As it stands now, the recent upward trend in rates of *prevalence* cannot be directly attributed to an increase in the *incidence* of the disorder. Most of the existing epidemiologic data are, however, inadequate to properly test hypotheses on changes in the incidence of PDD in human populations. Moreover, due to the relative rarity of autism and PDDs, power is seriously limited in each investigation, and variations of small magnitude in the incidence of the disorder would be very hard to measure.

Numbers of Affected Children in the United States

Using population estimates for the United States in the year 2000 and estimates of 30/10,000 (or 60/10,000, respectively), it can be estimated that about 220,000 (483,000) subjects under the age of 20 years have a PDD in the United States.

Conclusion

Epidemiologic surveys of autism have now been carried out in several countries. Methodologic differences in case-definition and case-finding procedures make between-survey comparisons difficult to perform. In spite of these differences, some common characteristics of autism and PDDs in population surveys have emerged with some consistency. Autism is associated with mental retardation and is overrepresented in males (with a male:female ratio of 4.4:1). Autism is found in association with some rare and genetically determined medical conditions, such as tuberous sclerosis. Autism is found in all social classes. The little evidence that exists does not support the hypothesis of secular changes in the incidence of autism, but power to examine and detect time trends is seriously limited in existing datasets. The debate has been largely confounded by a confusion between prevalence and incidence. Although it appears that prevalence estimates have gone up over time, this increase most likely represents changes in the concepts, definitions, and awareness of autism spectrum disorders in both the lay and professional public. To assess whether the incidence has *also* increased, method factors that account for an important proportion of the variability in rates must be tightly controlled. Taking 10/10,000 as the base rate for autism, a conservative rate of 27.5/10,000 for the combination of all PDDs can be derived. It could well be that, because these surveys were not focusing primarily on the nonautistic group, the actual rate of combined PDDs could be even higher, in the neighborhood of 60/10,000 to 70/10,000, as suggested by three recent surveys.

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2

Size of the Head and Brain in Autism: Clue to Underlying Biologic Mechanisms?

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In his original description of autism, Kanner (1943) noted that some affected children had relatively large heads. Indeed, "macrocephaly appears to be the single most consistent physical characteristic of children with autism" (Stevenson et al., 1997). Relatively large head size in autism is especially striking because a majority of persons with autism are also cognitively impaired, and cognitive impairment is commonly associated with heads that are smaller than population norms (Broman et al., 1987). The recent publication of several relevant studies makes it timely to review the current status of knowledge of the size of the head and brain in autism, a topic that may provide clues to the underlying neurobiology of this disorder.

Head Circumference

Head circumference, examined retrospectively in persons with a later diagnosis of autism, is generally normal at birth, as observed both in clinical samples (Lainhart et al., 1997; Stevenson et al., 1997) and in a case-control study within a population (Hultman et al., 2002). Measured after the newborn period, fronto-occipital head circumference (HC) tends to be larger in children or adults with autism than in the general population: 20–30 percent of autistic subjects had head sizes more than two standard deviations larger than those of the normal comparison group (Davidovitch et al., 1996; Woodhouse et al., 1996; Lainhart et al., 1997; Gillberg and de Souza, 2002). Stevenson and colleagues (1997) reported that HC exceeded the control mean in 80 percent of autistic subjects, and Courchesne et al. (2001) observed that 90 percent of autistic boys less than five years old had brain volumes on volumetric magnetic resonance imaging (MRI) that exceeded the mean for controls.

Conversely, in a population-based study of boys who underwent initial examination at about 8 months of age, big-headed infants were more likely than those

with normal-sized heads to have a later diagnosis of autism spectrum disorder (ASD) (Bolton et al., 2001). As in other studies, macrocephaly was more frequent among infants of higher socioeconomic status. Adjusted for socioeconomic status, ASM was more than five times more frequent in macrocephalic than in normocephalic boys (AOR: 5.6; 95% CI: 1.1–54.6; $p = 0.03$). Many studies have indicated that in autism, head size was not associated with mental retardation, seizures, or other clinical subcategories. In a study that did not exclude children with other specific neurologic disorders, however, there was a bimodal distribution of head size, with a microcephalic group in which cognitive limitation was frequent and severe (Fombonne et al., 1999).

Factors Complicating the Interpretation of HC Data in Autism

Confidence in the association of large HC with autism as a basis for biologic hypothesis is somewhat impaired by weaknesses in the available studies. Most of the HC data come from clinical samples whose generalizability is uncertain. Studies have differed widely in the composition of the autistic subjects—by age, degree of cognitive handicap, proportion with other morbidities—and the comparison groups employed. Most older studies of head size (see below) used external, nonconcurrent, commonly old, norms that may differ from the cases measured with respect to ethnic group, gender, somatic size, and sociocultural factors, all of which are associated with variations in HC. It is not clear for most of these studies whether the comparison groups are entirely comparable.

HC and brain size are closely related in young children, but after the age of about 7 years, variations in the thickness of the skull and soft tissues attenuate that relationship. The ideal age at which to make case-control comparisons is, therefore, in early or middle childhood, but many existing studies of head size in autism have included some or a majority of adults. Importantly, the presence and severity of cognitive impairment and of other medical disorders has varied in existing studies. Some comorbid conditions tend to be associated with aberrant head size (e.g., congenital rubella with microcephaly).

First-degree relatives of persons with autism, not themselves autistic, tend to have larger than average head size (Fidler et al., 2000; Miles et al., 2000). HC may thus be related to family characteristics that are not necessarily closely associated with autism. Persons with autism tend to be taller than the general population (Davidovitch et al., 1996; Lainhart et al., 1997; Stevenson et al., 1997; Miles et al., 2000) and autistic persons with big heads tend to be taller than autistic persons with smaller heads (Davidovitch et al., 1996). Only a few studies have examined the proportionality of HC to other measures of somatic size.

As Kanner noted (1943), parental education and income tend to be relatively high in families of autistic persons. Although referral bias could account for this association, Kanner did not observe such a trend in children in his medical practice who had other diagnoses. The largest study of socioeconomic factors in autism to date, a recent population-based study with a denominator of 4.6 million live births (Croen et al., 2002), observed a fourfold higher rate of autism in children whose mothers had had postgraduate education, compared with those who had not completed high school. Adjusted for race, birth place, maternal age, birth order, and other factors, the excess of autism in highly educated families was twofold. Because height, socioeconomic status, and HC (and, outside of autism, IQ) are correlated, it is not yet excluded that head size in autism may be appropriate for the larger somatic size and higher education and socioeconomic status that may tend to accompany autism.

Brain Size Assessed by MRI

Five recent studies have contributed to our knowledge of brain size in autism. In *nonmentally retarded persons with autism, chiefly adults,* well matched for intellectual level and social factors, Hardan et al. (2001) observed that intracranial and brain volume on MRI were slightly larger in the 16 persons with autism compared with 19 controls. The third ventricle was larger in autistic persons. Adjusted for intracranial volume, cerebral volume was significantly larger in autistic subjects and controls younger than 21 years, but not in older groups.

In a study of *ASD with or without mental retardation in young children and adolescents,* Courchesne et al. (2001) performed MRI volumetric studies in 30 autistic boys aged 2–5 years and in 12 control boys of similar age recruited through community advertisements. They also studied older boys, 30 of them autistic, and 40 controls, aged 5–16 years. IQs in the control children of all ages were relatively high (verbal IQ: 80–132; nonverbal IQ: 90–140), whereas the IQs of the entire autism group ranged from 40 to 90. The intellectual level of the autistic subjects and controls less than 5 years old, the critical comparison groups, was not described, and there was no consideration of the possible relationship of brain size to IQ in the results of this study, or of somatic size or socioeconomic factors. Below the age of 5 years, 90 percent of the 30 autistic boys' whole brain volume and cortical gray matter exceeded the mean for the 12 control subjects. In 2- and 3-year-old autistic boys, there was 18 percent more cerebral white matter than in control boys of the same age group ($p < 0.001$), and cortical gray volume was 12 percent greater than in controls of similar age ($p = 0.001$). In the older autistic boys, however, there was little difference from controls in brain size and, indeed,

brains in the adolescent control subjects were somewhat larger than those of adolescents with autism.

McAlonan et al. (2002) used quantitative MRI to study *adults with Asperger syndrome,* with a sample of 21 affected persons and 24 controls. IQs were all in the normal range, although slightly below average in some cases. Total intracranial volume was not different in these adults with Asperger syndrome, compared with controls, but there were significant reductions in gray matter volume in frontostriatal and cerebellar regions and white matter excesses around the basal ganglia, with white matter deficits elsewhere, especially in the left hemisphere. In addition to these complex observed differences, adults with Asperger syndrome did not demonstrate the age-related reduction in volume noted in controls. Height and social status were not described.

Aylward et al. (2002) compared *autistic children and adults without mental retardation* and controls. IQs were slightly lower in some autistic subjects, but height and social status were comparable. Brain volume was larger in the 23 autistic subjects younger than 12 years of age, compared with the 28 controls, that difference not reaching conventional levels of statistical significance without control for height, but being significant when controlled for height. Externally measured HC was larger and the distribution was unimodal, both in children and adults with autism. In subjects older than 13 years, brain volume on MRI was comparable in autistic cases and controls. Brain volume of adolescents with autism was slightly smaller than in younger autistic children less than 12 years of age, whereas brain volume of control adolescents was slightly larger than in younger control children.

Sparks and coworkers (2002) investigated *ASD with or without mental retardation, compared with normal or developmentally delayed children,* in children aged 3–4 years. Developmental delay was defined according to Vineland norms and using the Mullen Scales of Early Learning, with respect to which autistic and delayed children were comparable. Controls were excluded if they scored more than 1 standard deviation above or below the norm on the Vineland Adaptive Behavior Scales. On MRI, both cerebrum and cerebellum of the 45 children with autism were larger than those of 26 normally developing and 14 developmentally delayed children. There was no evidence of a bimodal distribution of brain volume among the autistic children.

Of the recent cross-sectional studies of brain volume in autism cited, two have examined young children aged less than 5 years, at a time when the pathobiology might be relatively active. The disadvantage of this strategy is that children suspected of autism early may tend to be those with serious language or intellectual abnormalities, so the relationships of brain findings to those deficits need to be considered. However, three studies have compared non–mentally

retarded individuals, with and without autism (although IQs in persons with autism tended to be lower than in controls); such comparisons were made, perforce, in older individuals. The observation that fairly comparable results emerged from all these studies strengthens the evidence that early brain growth is aberrant in autism.

Boys with autism have been noted to grow in height more rapidly than do controls, with earlier cessation of growth (Katoda, 1991), suggesting a speeded-up developmental program for height, perhaps comparable with that for brain growth. Although it is not yet entirely clear whether larger somatic size and higher social status in autistic subjects (overall or in the clinical samples investigated) might not be sufficient to account for the brain and head size differences, the evidence that there is an early acceleration of brain growth makes this less likely. Sample sizes in studies to date are small, however, and the samples are highly selective. There is still a need for more comparability of case and control groups and further exploration of other variables associated with differences in brain size. The question of head and brain size in autism will not be fully resolved until there is a longitudinal study of head size across early childhood—a period during which brain size and head size are closely related—in a representative sample of autistic children. The preponderance of current evidence indicates that heads and brains of persons with autism tend to exceed population norms during early childhood, and this appears to be a shift of the distribution overall rather than the presence of a distinct large-brained subgroup. Differences in brain volume between cases and controls are apparently maximal in early childhood and may disappear thereafter. The microscopic anatomy of autism also changes with age after early childhood (Kemper and Bauman, 2002).

Brain Size in Other Disorders

In several other disorders characterized by relatively large head size but intellectual limitation, including fragile X syndrome and tuberous sclerosis, autistic features are relatively common (Fidler et al., 2000; Fombonne, 2000). In Down syndrome and Rett syndrome, head size is fairly normal at birth but falls below, and fairly dramatically below, population norms thereafter. In Down syndrome, there are reductions in dendritic spines (Becker, 1991) and a postnatal decline in pyramidal neurons of the prefrontal cortex that begins in the early months of life (Vuksic et al., 2002). In Down syndrome, a region of a specific chromosome is known to be involved, and yet the mechanism of cortical change (and of intellectual impairment) remains unknown. Down syndrome is an attractive subject for the study of factors relating to brain size and cognitive limitation, because it is clinically recognizable at birth (as autism is not), independent means are avail-

able for early diagnosis at or before birth, and experimental models for research purposes exist.

Head size and brain weight, near normal at birth, depart markedly from norms in Rett syndrome (Armstrong, 2001). Mutation of a single gene, *MECP2,* is involved in most cases of Rett syndrome, but that mutation has a destabilizing effect on chromatin structure, so that the accessibility to regulation of several genes is altered (Dragich et al., 2000). Transcription factors operate in combination, so that a given factor may take part in regulating many genes. Aberrations in brain growth may not be easy to link to single-gene effects. In an animal model, prenatal infection with influenza virus can produce behavioral abnormalities, neuronal size changes, and relatively large head size (Fatemi et al., 2002).

Molecular Pathogenesis of Autism

Much of the attention to biologic mechanism in autism has centered on neurotransmitters, especially serotonin and gamma-aminobutyric acid (GABA). Neurotransmitter function develops relatively late in prenatal life, but there may be reasons for focusing neurochemical research on even earlier processes. For example, these same molecules, serotonin and GABA, also function much earlier in gestation as trophic factors (Kriegstein and Owens, 2001; Whitaker-Azmitia, 2001). In addition, brain-derived neurotrophic factor (BDNF; see below) is a trophic factor for serotonergic neurons (Madhav et al., 2001) and influences the release of serotonin, as well as GABA and dopamine (Goggi et al., 2002).

We focus here on issues related to brain size in autism, asking why autistic brains grow faster than normal in the first years of life. Why do certain neuronal types fail to survive, and why do some brain structures have an apparent excess of neurons? The net growth of the brain early in normal development is due to the balance between two vigorously competing processes, one of them progressive—including the increase in numbers and size of neurons and glia, and the increase in synaptic contacts between neurons—and the other regressive and including programmed death of neurons and pruning of synapses. The number of neurons is largely determined in the second half of the second trimester of prenatal life, the number of glia thereafter. A rapid increase in synapse number and neuronal complexity and connectivity occurs during the first month to a year after birth. Following this period of increase, there is a much slower but very substantial period of cellular and synaptic loss that continues into the adolescent years (Huttenlocher and de Courten, 1987). During the first 1–3 years after birth, both growth and reduction proceed concurrently.

An increasing amount of information is becoming known about the processes, progressive and regressive, that govern brain and somatic growth and about

the molecules that control these processes. Although it is premature to guess as to which of the potential mechanisms for generating brain size and clinical manifestations are operative in autism, we offer two examples of how certain brain growth factors, identified in one study as aberrant early in the life of a group of children with autism (Nelson et al., 2001), might influence brain growth and development. A prototypical example of a molecule capable of influencing brain size, dendritic morphology, and behavior is the neuropeptide vasoactive intestinal peptide (VIP). The effect of VIP on early brain and somatic growth is dramatic (Gressens et al., 1993). One locus of action of VIP is on the duration of the mitotic cycle in neuroblasts (Gressens et al., 1998), its actions potentially altering the number of neurons that are born during the period of neurogenesis; thereafter, VIP influences the numbers of glia produced (Zupan et al., 1998). Early perturbations in VIP action, even brief perturbations, produce long-term effects (Wu et al., 1997). A highly active molecule secreted by astroglia on stimulation by VIP influences glutamate receptor development and dendritic structure in the hippocampus (Blondel et al., 2000).

A neurotrophin whose action is related to VIP is BDNF, which has effects on some of the cell types altered in autism, such as the cerebellar Purkinje cell (Ritvo et al., 1986; Larkfors et al., 1996). Global effects of BDNF on brain growth and synaptic circuit development have been demonstrated, and detailed mechanisms of action on the machinery of neurotransmitter release have been delineated (Pozzo-Miller et al., 1999). The expression of BDNF is specific to certain brain regions and neuronal cell types. Within the hippocampus, BDNF plays an important role in memory and in fear-motivated learning (Alonso et al., 2002).

Less satisfactory is the state of knowledge as to the molecular basis of synapse and neuronal elimination. The normal process of plastic development in an experimental system, the ocular dominance columns in the visual cortex, involves extensive synapse loss, which is prevented by overexpression of BDNF (Cabelli et al., 1995). This suggests that limiting amounts of trophins in a competitive developmental context could be responsible for some degree of synapse loss. Synapse loss can occur even in the presence of high levels of trophic materials, however (Nguyen et al., 1998).

Another powerful regulatory system shown to be responsible for normal activity-dependent synapse elimination, at least at the neuromuscular junction, involves the serine/threonine protease, thrombin, and its associated protease-activated receptor (PAR). The proteolytic activity of thrombin activates the PAR, which in turn couples into a number of cell biologic regulatory pathways, including activation of protein kinase C (PKC) (Jia et al., 1999). Both the trophin/peptide and the protease/PAR systems are coupled to a variety of kinases, and phosphorylation events mediate many of their actions. Knockout of a PKC

isoform produces striking persistence of polyneuronal innervation of the climbing fibers to Purkinje cell synapses in the cerebellum in experimental animals (Kano et al., 1995). Kinase-mediated phosphorylations can produce acute modification of synaptic efficacy, as well as activation of transcription factors such as cyclic AMP response element binding protein (CREB), to modify long-term gene expression.

How much difference in brain size might be accounted for by defective mechanisms related to synapse elimination, whether resulting from aberrations in trophin/peptide or thrombin/PAR systems or other pathways? Synaptic boutons constitute about 10 percent of gray matter volume (Chklovski et al., 2002), so even complete failure of the normal 40 percent synapse loss would produce only a 4 percent increase in gray matter volume. That is significantly less than the difference described between brains of young autistic persons and controls (Courchesne et al., 2001). Loss of synaptic connections between neurons and their targets would have other consequences; however, the resultant withdrawal of trophic input may lead to the death of neurons deprived of their trophic support (Perry and Cowey, 1982). The combined volume of synapses, axons, and dendrites makes up some 70 percent of the total gray matter volume (Chklovskii et al., 2002), so any alterations in their developmental trajectory would significantly alter overall brain size. An increase in white matter volume and, to a lesser extent, of gray matter, as seen in autism, might thus reflect a diminution in normal synaptic pruning and therefore excess survival of synapses and connecting neuronal cell bodies (in gray matter) and their axons (white matter).

As discussed recently with regard to other childhood psychiatric disorders (Gogtay et al., 2002), information on patterns of brain growth may aid in the understanding of disease pathophysiology. A great deal of information has become available regarding the mechanisms that influence growth and differentiation of neural tissue; for example, at least 20 factors have been identified that affect spinal motor neuron survival in vitro (Oppenheim, 1996), and a protein that regulates cerebral cortical size has been described (Chenn and Walsh, 2002). However, it is not known and is difficult even to guess, at this stage, which of these mechanisms are operative in complex human disorders, such as autism. The very richness and diversity of the regulatory processes have made it difficult to generate an inclusive and comprehensive scheme to describe the actual development and regulation of the intact nervous system. Normal function undoubtedly involves the interaction of multiple regulatory components, a prime example of the "biology of complexity" that still eludes complete analysis. Testable hypotheses relating laboratory observations to human disease are greatly needed.

The goal of achieving full understanding of the dysregulation of cerebral development that underlies autism is, unfortunately, some distance away, but

turning attention to factors that govern the development of the brain is likely to put us on the pathway toward increased knowledge and, it is to be hoped, toward strategies for remediation.

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3

The Autistic Mind

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This chapter focuses on two main issues: the processing and encoding of incoming information, and emotion and thought in autism. Although I refer to research findings, much of what I say comes from personal accounts (published and not) and from my clinical experience with adolescents and adults with autism. My thesis is that attention, when focused, is overly narrow; that this has major implications for how information is perceived, processed, and encoded in memory; and that emotion is connected to thought but with limited self-awareness. Throughout, I use the term *autism* to refer to the disorder of all those who, in varying degrees, fall on the autistic spectrum. However, the extent to which my arguments might apply to particular individuals will vary with both their cognitive status and the severity of autism.

Processing and Encoding of Information in Autism

The mind has been likened to a "filing system" consisting of multiple filing cabinets in which incoming information is organized. Waleski (personal communication, October 1997) extended this metaphor to people with autism, arguing that in them, minds are distinguished by two unique features. First, an obstruction exists between the senses and the mind, or, put somewhat differently, the door to the outside (sensory) world is blocked. Implied here is that incoming information may be incomplete or otherwise distorted. Second, information is stored in separate rather than conceptually or semantically related folders, thus resulting in precision at the expense of categorization and generalization. From the first, namely, that in autism, the senses obstruct the mind, I argue that hypersensitivity to sensory input results in states of overarousal and the adoption of an overly narrow beam of attention. Further, this narrowly focused attention is associated with acute perception, an analytical-computational and sequential

approach to processing information, and a precise but context-dependent memory system. Below I elaborate on each in turn.

Evidence suggests that in autism, the physical or perceptual world has an unusually strong pull. It is as if sensory information is too salient. Speech, for example, may be perceived as overly distinct phonemes rather than segmented into words, which (unlike phonemes) denote meaning. Anecdotal reports of people with autism implicate a lower threshold for perceiving sounds (e.g., a train in the far distance; Cesaroni and Garber, 1991) or visual phenomenon (e.g., particles in the air; Williams, 1992), and underscore that various forms of sensory stimulation may be experienced as unusually intense or even painful (Grandin, 1986). Hypersensitivity to sensory stimulation, whether novel, unpredictable, or otherwise experienced as intense or disturbing, may induce a state of overarousal (cf. Dawson and Lewy, 1989), such that the mind is obstructed by the senses, and information uptake is incomplete or even distorted. In an attempt to offset states of overarousal, information uptake may be further restricted by the adoption of an overly narrow focus of attention (cf. Zentall and Zentall, 1983).

Consistent with such claims, empirical findings have been taken as evidence of "tunnel vision" (Rincover and Ducharme, 1987) or of a narrow beam or "spotlight" of attention in autism (Bryson et al., 2004; Landry and Bryson, 2004). Even under relatively benign viewing conditions, people with autism have marked difficulty disengaging attention from one stimulus or event and moving it elsewhere: they remain "stuck" on one of two competing stimuli (Bryson et al., 2004; Landry and Bryson, 2004). Indeed, failure to respond to their names, once absorbed or focused on a particular activity, is one of the best early indicators of autism (Robins et al., 2001). Evidence of overly focused attention also comes from studies of visual discrimination learning, in which people with autism tend to respond to only one of multiple stimulus dimensions (e.g., color versus form; Rincover and Ducharme, 1987). Such effects have been found to distinguish autism when the (spatial) distance between stimulus dimensions is increased, thus underscoring that the beam of attention is unusually narrow.

One implication of a narrow beam of attention is that incoming information will be perceived as brighter or louder, or as otherwise more salient. Such heightened perception would contribute even further to the pull of the physical world, and is consistent with anecdotal reports of acute attention to and memory for perceptual detail. As might be expected, evidence indicates that people with autism are actually superior to the norm at detecting a figure embedded in a complex visual display (Shah and Frith, 1983). The same is the case on complex visual search tasks requiring the detection of an anomalous feature (O'Riordan et al., 2001; also see Bonnel et al., 2003, for evidence of superior auditory dis-

crimination). This enhanced perception in autism suggests that the mental representations or memory codes for patterns or objects are more readily activated than is normally the case (Brian et al., 2003). In the case of action, this is well exemplified in tasks requiring that objects be used for purposes other than the familiar (e.g., hammering with a toothbrush). People with autism have difficulty inhibiting the well-practiced actions associated with particular familiar objects (Smith and Bryson, 1998).

Overly focused attention in autism appears to be associated with a reliance on an analytical and computational approach to processing incoming information. An analytical (versus holistic, gestalt, or synthetic) processing style is thought to underlie performance on tasks requiring the reconstruction of block designs, on which superior performance has been documented in people with autism (Shah and Frith, 1993). Their approach in these and related tasks (e.g., drawing) appears to be one of building up piece-by-piece to the whole (Sacks, 1995). By contrast, their ability to see or apprehend the whole in the parts from the outset, as required in skillfully assembling puzzle pieces of familiar objects, is less well developed (see Frith, 1989, who argues for the lack of "central coherence" to explain this and related phenomena). People with autism also fail to show the McGurk effect (McGurk and MacDonald, 1976); that is, the tendency for speech perception to be influenced by visual information (for a "d" sound to be perceived as "p" when the mouth makes a "p" movement; Jessel, 1995). Their attention is overly focused on the auditory (versus visual) information, and/or they have difficulty integrating or synthesizing the two sources of information. Several individuals with autism have reported to me that it is easier to follow what others are saying by focusing on their speech rather than looking at them, as if looking actually interferes with an analysis of what is heard.

Interest and talent in such domains as music, languages, mathematics, and computers would also argue for an analytical processing style in autism. Indeed, some individuals are unusually skilled at penetrating the underlying structure or logic of formal or physical systems. One relatively capable person who attended university was described by a professor as having an "uncanny" sense of the syntax (versus semantics) of multiple languages. Similarly, computational skills, as required not only in mathematics or computing dates, but also in drafting or drawing to size (e.g., Wiltshire, 1991), can be truly remarkable, even in less capable people with autism. One such person whom I know well cannot cope with the spatial aspects of a basketball court or multiple moving players but is exceptionally skilled at computing the operations necessary to accurately place basketballs in a net (and therefore he remains stationed near the net and others simply pass to him!).

This approach to the world also appears to be associated with a tendency toward processing information sequentially rather than simultaneously or in parallel. Assuming, as the evidence indicates, that attention is overly focused in autism, information would, by necessity, be processed sequentially. A reliance on analysis or logic would suggest the same. In those capable of at least some understanding of the minds of others, a more analytical and presumably sequential processing style is adopted. One person with Asperger syndrome, hurt by the accusation that he lacks sensitivity to others, described the process as lengthy, involving a series of "if this, then that" computations. I would add, however, that in people with autism, this processing style is not specific to the social realm, but rather, characterizes their approach to most forms of information. It may simply be that the complex, ever-changing, and multidimensional nature of social information places particular limitations on the effectiveness of a more analytical and sequential processing style (cf. Dawson and Lewy, 1989).

Consistent with an overly focused and analytical or restricted processing style, people with autism tend to file information in separate rather than conceptually or semantically related folders (Waleski, personal communication, October 1997; also see Luria, 1968, for a rich description of a mnemonist, and Hermelin and O'Connor, 1967, 1970, for relevant discussions). This results in precision at the expense of categorization and generalization: memory for specific events (versus their relationship to previous experience) is relatively strong, although retrieval of information tends to be context-dependent (i.e., dependent on the presence of particular stimulus cues). Similarly, actions would appear to be coded in overly specific but precise ways (Smith and Bryson, 1994). Such a system might explain why people with autism are able to access information in response to specific rather than general questions, or why they produce topographically similar responses in the presence of the same but not similar stimulus conditions.

Overly specific and more readily activated memories might also underlie a preference for facts rather than fiction. Apprehension of the directly accessible physical or perceptual (versus mental) world is well documented in people with autism (e.g., Baron-Cohen et al., 1986). They have a relatively good sense of physical causality and of how things fit together. Some individuals report that they think in pictures, with difficulty conceiving of categories such as "dog," as there is no categorical referent, only specific (different-looking) referents for each type of dog (e.g., Grandin, 1995). Similarly, relational terms, such as pronouns, prepositions, and some adjectives (e.g., large), pose a problem, as there are no constant or context-independent referents (Park, 1967; Menyuk and Quill, 1985). Memory appears to be true to the facts rather than reconstructed by thought and experience. However, many individuals with autism develop at least some imaginary

thought, however repetitive or routinized. Indeed, as in typical young children, they may become absorbed in such activities, have difficulty distinguishing between reality and fantasy, and be quite insistent that their fantasies are true, at least in part because their images are so compelling. My experience suggests further that their capacity for absorption may be associated with the presence of imaginary friends, the co-occurrence of which has been documented in a subset of typical young children who appear prone to hypnotic states (Hilgard, 1979). It may be, as implied by Park (1967, 2001), that the "siege" involves a journey from a relatively safe, inner-centered, and trancelike state of "nirvana" to the outside world of people, as well as of things.

Emotion and Thought in Autism

Emotion, like thought, is a complex issue in autism. There are at least three defining features of emotion. These include its expression, the physical sensations associated with feeling states, and the cognitive meaning we attribute to emotional experiences (Plutchik, 1984). People with autism tend to be emotionally nonexpressive, whether indexed by facial emotion or the use of eye movements, gestures, or vocal intonation (e.g., Yirmiya et al., 1989; also see Czapinski and Bryson, 2003). It bears emphasizing, however, that a lack of expressed emotion does not necessarily imply a lack of felt emotion. Indeed, contrary to what conventional wisdom might suggest (e.g., Kanner, 1943), I would argue that people with autism experience a range of emotions, particularly the primary emotions (happiness, sadness, surprise, fear, and frustration/anger), although the expression of these may not be readily recognized. Rather, in autism, emotion tends to be expressed atypically (e.g., through hand flapping), or in ways more typical of very young children (e.g., excessive mouthing, gaze aversion, or physically moving away; Landry, 1998).

In my experience, the most striking exceptions occur under conditions of great personal significance, in which the emotion experienced is extreme. In one instance, a relatively capable 18-year-old with classic autism was confronted with the death of his girlfriend, with whom he had spent considerable time and had reported feelings of "love." On hearing the news, he broke down, appropriately sobbing and embracing his father in a way that had never been observed in him by either of his parents. Parents of a younger child provide a similar description of typical, coordinated affect expression when their son was saved from drowning by his father. In another youngster (aged 16), also with classic autism but less capable linguistically, I directly observed an incident in which he was clearly very angry, ostensibly about being "bossed around." With the exception of his language, which was surprisingly fluent but odd (yelling, "You are nothing but a

noun, and you a verb"), all aspects of his behavior, including facial expression, the look in his eyes, stance, and use of gestures and intonation, expressed anger in a normal, integrated fashion.

However, extreme emotion in individuals with autism can also result in catatonic-like freezing (cf. Wing, 2000), underscoring that the window of normal affect expression (like attention) may be unusually narrow. Examples I have observed include a woman who, hearing of my father's death, stood frozen for several minutes with her arm in the air and a look of absolute terror. Later in the day, on her own accord, she bought and hand-delivered the most emotion-laden sympathy card that I received. I have observed similar reactions (i.e., initial "freezing" followed by expressions of concern) in adolescent boys who had just learned that one of their housemates was missing and could not be located. My sense is that in autism, the internal physical sensations associated with emotion can be experienced as intense and even overwhelming, and, as with other forms of sensory stimulation, may result in a state of overarousal. Overarousal will presumably only exacerbate existing problems, both in differentiating feeling states and in regulating emotion. Indeed, even at the best of times, many people with autism seem capable of distinguishing only between "good" and "bad" feelings. In the extreme, good feelings may readily become aversive, complicating matters further.

My main point is that, regardless of difficulties in expressing emotion and in distinguishing subtle differences in their own feeling states, people with autism do experience a range of emotions. Despite their well-documented difficulties reading emotion in others (e.g., Hobson, 1986), many have at least some sensitivity, although typically, this is based on distinct physical cues, such as tone of voice rather than the more subtle facial and gestural cues so communicative for most of us. One child with Asperger syndrome whom I know well becomes noticeably distressed and actually asks his mother "to use her loving voice" when she is angry or otherwise upset. Adolescents and adults can be particularly sensitive to criticism and rejection, although they may protest indifference or respond with anger, which, in my experience, may provide an escape from their pain of hurt. Indeed, I know individuals with autism who actively defend against the softer emotions, specifically those that elicit crying, which they report "feels terrible and uncontrollable." When unavoidable, one woman takes a shower, and lets the water cry for her.

Finally, and again contrary to conventional wisdom (Hermelin and O'Connor, 1985), I would argue that in autism, emotion is connected with, although poorly modulated by, thought. The co-existence of the two is well exemplified by the tendency to avoid particular situations or people because of incidents associated with negative emotion. The intensity of the emotion, as

revealed in the person's retelling of the incident, can be truly remarkable, particularly given that the event may have occurred many years in the past. I know a 42-year-old woman with classic autism who was distressed by the intensely negative emotion she routinely experienced when visiting her parents. Together, we came to understand this as an association formed while staying with them following hospitalization for a psychotic break, which she experienced as devastating. She is now able to enjoy her parents by visiting with them in places other than their home.

The presence of the more cognitively complex emotion of guilt in at least some people with autism also serves to underscore that emotion is connected to thought. However, emotion is poorly modulated by thought. This most likely reflects the intensity of the emotion, as well as the lack of self-awareness typical of most people with autism (see Frith and Happe, 1999, for a detailed discussion). The ability to reflect on the self depends on what may be common to most if not all high-order executive functions; namely, the capacity to suspend immediate experience, whether direct perception or a thought arising from perception, in order to think about that which is being experienced (Dennis, 1991; also see Russell, 1997). Evidence indicates that executive tasks pose a problem for people with autism (Ozonoff, 1995). Rather, it appears that they have difficulty disengaging from emotionally significant events or thoughts, and that these can become the focus of prolonged and obsessive or anxiety-driven attention. In my experience, reasoning with them is difficult, as their attention is overly focused on the event in question, and once fixated, it is extremely difficult for them to disengage and think about anything else. The potential significance of this is underscored by claims that the disengagement operation plays an important role in the regulation of emotion (Rothbart et al., 1992). Perhaps the most basic way we deal with emotional upset is by disengaging or distracting ourselves from the source of distress. In people with autism, difficulties disengaging attention may place them at increased risk for intense and prolonged emotional upset.

Summary and Conclusion

My intent here has been to develop some ideas about the mind in people with autism. Two issues have been addressed: the processing and encoding of information in memory, and the relationship between thought and emotion. I have argued that in autism, the mind is vulnerable to sensory overload, that it is hypersensitive to sensory stimulation, and that it adapts to the resulting state of overarousal by assuming a narrow beam or focus of attention. Although this limits the boundaries of perception, information within the narrow focus is perceived as unusually intense and thus imprinted strongly in memory. Overly

focused attention in autism appears to be associated with an analytical-computational and sequential style of processing information. Information is approached in a piecemeal fashion, in which the "whole" is systematically built up or constructed from the parts in sequence. In contrast, the ability to process multiple sources of information simultaneously or in parallel, as required, for example, in seeing the "whole" from the outset, appears to be a relative weakness. In my view, such difficulties are particularly obvious in but not specific to the social domain (e.g., in reading the emotions of others). I have argued, further, that the more focused and analytical or restricted processing style in autism results in an overly precise and context-dependent memory system. Incoming sensory information appears to be filed in separate rather than conceptually related folders. Memory for the details of events is relatively strong, but the costs include a lack of categorization and generalization, and a reliance on specific contextual cues to access information from memory.

I have also argued that people with autism experience a range of emotions, particularly the primary emotions, and that, like other sources of sensory stimulation, emotion can be experienced as intense and overwhelming. My main point is that it is important to distinguish between the experience of emotion and other aspects of our emotional lives. Clearly, the expression of emotion poses a problem for people with autism, as does their difficulty detecting and understanding emotion in others. However, none of this implies that they themselves do not feel, or that they have no sensitivity to emotion in others, or that thought is necessarily disconnected from their emotional states. Rather, I have argued that in autism, emotion is connected to but poorly modulated by thought, and that such difficulties most likely reflect both the intensity of experienced emotion and a lack of self-awareness (i.e., the ability to reflect on one's thoughts or experiences). When people with autism are aroused by emotionally significant events, their attention becomes narrowly focused, the tendency to engage in repetitive behavior is enhanced, and it is extremely difficult for them to disengage and move their attention elsewhere. Not surprisingly, under such circumstances, positive emotion can readily be experienced as aversive.

Two former students and I have previously suggested that the phenomenon of hemispatial neglect might provide a useful framework for thinking about the emotional and cognitive lives of people with autism (Bryson et al., 1990; also see Ornitz, 1989). Briefly, their pervasive inattention to and lack of awareness of both external stimulation and their own internal states serve to underscore the parallels between the two (cf. Heilman et al., 1979). However, what we know about spatial neglect comes from studies of adults who have suffered an injury to the brain, whereas autism is, of course, a disorder of development. We can there-

fore only speculate about the implications of spatial neglect for a developing child. It would appear, for example, that in autism, information outside the narrow focus is processed preattentively or automatically. A failure to actively process figure and ground, such that the two are decoupled and related to previous experience, might underlie their context-dependent learning. Similarly, inattention to and lack of awareness of internal states, together with states of overarousal and extreme anxiety, would presumably have major implications for the development of a sense of self, as well as that of others. Future research on autism might profitably explore these and related issues, using spatial neglect as a model for conceptualizing important outstanding questions.

In the meantime, there are both empirical and logical grounds for assuming that in autism, there is a narrow focus of attention, an analytical-computational and sequential approach to processing information, and a precise but context-dependent memory system. And, to the questions, "Do people with autism have an emotional life?" "Are they sensitive to some emotion in others?" and "Is their emotion connected to thought?" my answer is "Yes." I do not mean to minimize their expressive difficulties or their difficulties in detecting, making sense of, or regulating emotions, whether their own or those of others. However, my experience suggests that there is much for us to understand about the experience of emotion in autism: that many individuals are highly emotionally sensitive, that anxiety poses a significant problem, and that a better understanding of emotion, as well as of thought, will allow us to more effectively intervene and support the needs of the many individuals affected.

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4

Language and Communication Disorders in Autism Spectrum Disorders

Helen Tager-Flusberg, Ph.D.

One of the key diagnostic features of autism includes "qualitative impairments in communication" (APA, 1994:70). By definition, children with autism show delays and deficits in the acquisition of language, which range from the almost complete absence of functional communication to adequate linguistic knowledge but impairments in the use of that knowledge in conversation or other discourse contexts. In contrast, one of the striking aspects of the DSM-IV diagnostic criteria for Asperger syndrome, also considered on the spectrum of autistic disorder, is the absence of any language or communicative impairment. Over the past several decades, a considerable number of descriptive studies have been conducted on the nature of the language impairment in autism spectrum disorders (for recent reviews, see Lord and Paul, 1997; Wilkinson, 1998; Tager-Flusberg, 2000). These studies all focus on verbal children with autism, although it is important to note that perhaps half the population never acquires functional language (Bailey et al., 1996).

This chapter reviews the literature on the main features of language and communication problems in autism spectrum disorders, with special reference to identifying features that are universal across the spectrum and those that may be important for identifying different subgroups within autism. The final section reviews recent studies that have explored the neurobiologic substrate for language in children with autism, in an effort to relate brain structure to the clinical features of autism in this domain.

Clinical Features of Language Impairment in Autism

Parents of children with autism often report that the first sign of a problem with their child was either the absence of language or the loss of the language that had begun to develop in the second year of life (Kurita, 1985; Lord and Paul, 1997). By their first birthday, many infants who later receive the diagnosis of

autism do not respond to their own name and fail to make eye contact (Osterling and Dawson, 1994). By the end of the second year, almost all toddlers with autism still have no functional language and are extremely limited in their communication with others, perhaps engaging another person only to fulfill requests using limited pointing gestures (Stone, 1997).

To some extent, we see that the primary social deficits in autism set the developmental course for deficits in language and communication: there is little or no interest in interacting with others at any level or by any means, including language. Nevertheless, some children with autism, usually those who are less severely impaired overall, do increase the frequency of their communicative attempts and begin acquiring language before their fifth birthday. Acquiring some functional language by the age of five has been found to be the most powerful predictor of a more positive outcome in autism spectrum disorders (Rutter, 1970; Ventner et al., 1992).

Kanner (1943, 1946) was the first to note that children with autism would often simply echo the words, phrases, or sentences spoken by others. This classical feature of autistic language, known as *echolalia,* is most typical of children who have very little productive language (McEvoy et al., 1988). Echolalic speech often retains the exact words and intonation used by others either immediately or after some time. It is now viewed as having some functional value for autistic children. Echolalia may help children with autism to maintain some role in the ongoing discourse, even when they either do not understand or have not yet acquired either the pragmatic or linguistic skills needed to respond more appropriately (Prizant and Duchan, 1981; Tager-Flusberg and Calkins, 1990). Odd vocal patterns and monotonic vocal expression or prosody were noted in many early descriptions of autistic children's language, both in echolalic and nonecholalic speech (Pronovost et al., 1966). Kanner (1946) also noted the autistic child's tendency to use words with special or unique meanings, not shared by others. The use of idiosyncratic lexical terms, or "neologisms," has been found even in higher-functioning children and adults with autism (Volden and Lord, 1991) suggesting that, unlike echolalia, it does not mark a developmental stage in acquisition.

Another striking feature of autistic children's use of language is their reversal of pronouns—referring to themselves as "you" and their conversational partner as "I." These reversal errors are viewed as important in the diagnosis of this disorder (Le Couteur et al., 1989; APA, 1994). They reflect difficulties in conceptualizing the notion of self and other as it is embedded in shifting discourse roles between speaker and listener (Tager-Flusberg, 1993, 1994; Lee et al., 1994).

Autism has been identified as a language disorder that, at its core, involves pragmatic impairments, defined as deficits in the use of language in social con-

texts (Baltaxe, 1977; Tager-Flusberg, 1981; Lord and Paul, 1997). Children with autism are often unresponsive to the conversational initiations of others. Even when autistic children do engage and respond to others, they may offer little to the ongoing discourse, and have difficulty sustaining the conversational topic (Tager-Flusberg and Anderson, 1991). These discourse deficits are seen as central to the defining characteristics of autism (Tager-Flusberg, 1996, 2000).

Although current diagnostic criteria for Asperger syndrome do not include impairments in language or communication, Hans Asperger (1991) and others noted numerous clinical abnormalities in individuals with this disorder. Asperger noted that all the boys for whom he provided case histories had significant deficits in nonverbal social communication. Their vocal expression was deficient in expressive prosody, which is used to maintain social contact and convey affect. Other features noted by Asperger (1991) and confirmed by other clinicians include neologisms, pedantic speech, and an idiosyncratic approach to communication, including deficits in social discourse (Gillberg, 1989; Klin and Volkmar, 1997; Twachtman-Cullen, 1998; Landa, 2000), suggesting considerable overlap in these symptoms with classic autism.

Core Communication Impairments in Autism Spectrum Disorders

This overview highlights the emphasis on communicative impairments, especially in pragmatics and discourse, reflecting the language problems that are universal and unique to autism spectrum disorders (Tager-Flusberg, 1999). Systematic research investigations have been conducted in recent years to understand the core problems in this domain, especially vocal impairments and social discourse, and to explore their relationship to other aspects of social deficit in autism within a developmental perspective.

VOCAL IMPAIRMENTS

Because autism is never diagnosed during infancy, there are no studies of early vocal development in this population. There have, however, been a few studies on the development of speech sound production and articulation. Some controlled studies of children with autism report that phonologic skills are relatively unimpaired, and may even be precocious (Bartolucci and Pierce, 1977). According to detailed studies, phonologic errors produced by children with autism are similar to those reported in the literature on normal development, suggesting delayed but not deviant development in this linguistic domain (Bartolucci et al., 1976). By middle childhood, children with autism who develop functional language gener-

ally have mature phonologic systems; however, there are reports of a relatively small number of high-functioning children with autism who continue to have extraordinary difficulty producing intelligible speech (Lord and Paul, 1997). Little is known about these children, because they are typically not included in systematic studies.

The voice quality and intonation patterns of the speech of both children with autism and Asperger syndrome have been described as strikingly atypical, and these abnormalities appear to persist through adulthood. Numerous prosodic features have been noted, including monotonous speech (Lord and Rutter, 1994). Other children appear to have a more melodious singsong pattern of speech (Fay and Schuler, 1980), but it is equally devoid of communicating emotion or intent. Atypical pitch patterns have been documented in some children (Pronovost et al., 1966), and problems with both volume and voice (e.g., hoarseness, hypernasality) have been reported.

Even though these atypical speech patterns are very common and appear across the full range of the autistic spectrum, very little systematic research has been conducted into this unusual syndrome-specific abnormality, and so the explanation for these diverse speech characteristics remains obscure. Some have suggested that they reflect the pragmatic and social-affective deficits that are central to the syndrome of autism (e.g., Lord and Rutter, 1994; Tager-Flusberg, 1996). Interestingly, they are among the earliest symptoms to appear. Studies have found that mothers of prelinguistic toddlers and children with autism have particular difficulty interpreting the meanings conveyed by vocalizations produced by other children with autism, although they can understand their own children's messages (Ricks and Wing, 1976; Lord and Paul, 1997). Abnormalities in speech and voice may reflect the fact that infants with autism pay so little attention to the speech of others in their environment and, unlike other children, are not concerned about matching their social surroundings.

PRAGMATIC IMPAIRMENTS

Prelinguistic social-communicative development in infants with autism offers a striking contrast to other groups of atypical children. Many infants with autism are described as showing little or no interest in people, and some parents report, retrospectively, that it was difficult to maintain eye contact or engage in interaction with their babies (Ornitz et al., 1977). Prelinguistic toddlers with autism show no preference to listen to their own mothers' speech (Klin, 1991) and may have idiosyncratic means of conveying different needs, which their mothers find difficult to interpret (Ricks and Wing, 1976). These severe social deficits culminate in their well-documented problems in joint attention (Loveland and

Landry, 1986; Mundy and Sigman, 1989; Mundy et al., 1990, 1993). Longitudinal studies of young children with autism suggest that these early deficits in joint attention are predictive of later language development (Mundy et al., 1990; Sigman and Ruskin, 1999; Charman et al., 2003).

The move toward the onset of spoken language suggests that communication with others provides one of the key motivations for learning language. Wetherby and Prutting (1984) examined the range of speech acts that were expressed by children with autism in both gestural and spoken language at early stages of development, in comparison to language-matched normally developing children. They found that the children with autism were unimpaired in their use of language for requests for objects or actions, protests, and self-regulation (e.g., "Don't do that"). Yet certain speech acts were completely absent. These included comments, showing off, acknowledging the listener, and requesting information. These findings are consistent with several other studies (e.g., Loveland et al., 1988). The speech acts missing from the conversations of children with autism all have in common an emphasis on *social* rather than environmental uses of language (Wetherby, 1986).

A number of studies have investigated conversational skills in children with autism. Tager-Flusberg and Anderson (1991) reported significant differences between young children with Down syndrome and autism in their ability to maintain conversational topic while they were interacting with their mothers. The children with autism often did not respond in a topic-related way to their mothers' utterances; instead, they introduced irrelevant or repetitive comments. Even when the children with autism did respond on topic, they did not develop the capacity to expand or elaborate on the information provided by their mothers. Capps et al. (1998) replicated these findings in older children, who were engaged in conversation with an experimenter. Similar difficulties in social uses of language, especially in conversation and other discourse contexts, have been widely noted for people with Asperger syndrome (e.g., Ghaziuddin and Gerstein, 1996; Klin and Volkmar, 1997).

Some studies have investigated more advanced discourse skills—specifically, narrative skills—in higher-functioning autistic children. A detailed study conducted by Loveland and her colleagues compared groups of children with autism and Down syndrome in their ability to retell a story they were shown in the form of a puppet show or video sketch (Loveland et al., 1990). Compared to the controls, the children with autism were more likely to exhibit pragmatic violations, including bizarre or inappropriate utterances, and were less able to take into consideration the listener's needs. Some of the children with autism in this study even failed to understand the story as a representation of meaningful events, suggesting that they lacked a cultural perspective of the underlying narrative

(Bruner and Feldman, 1993; Loveland and Tunali, 1993). Similar findings were obtained by Tager-Flusberg (1995), and these impairments in producing stories are reflected in impairments in story comprehension in both autism (Norbury and Bishop, 2002) and Asperger syndrome (Joliffe and Baron-Cohen, 2000).

Research on narrative deficits in individuals with autism or Asperger syndrome suggests that some of the subjects' difficulties may lie in their inability to consider their listener's needs and current level of knowledge. For example, individuals with autism perform significantly worse than controls on referential communication tasks, in which they have to communicate about something only they know to a listener (Loveland et al., 1989). They have trouble responding to requests for clarification by providing additional information (Paul and Cohen, 1984). They also have difficulty judging the amount of information that needs to be included for effective communication (Surian et al., 1996). These deficits in conversational discourse and narrative, which persist in older and higher-functioning individuals, including those with Asperger syndrome, appear to be closely linked to social impairments that are at the core of the disorder of autism (Capps et al., 1998).

Across the spectrum of autism disorders, a particular profile of language impairment emerges from the studies reviewed here. Vocal communication and pragmatic skills are uniquely and specifically impaired across both autism and Asperger syndrome. Several studies have explored the potential overlap between autism and Asperger syndrome, and most conclude that this overlap is very significant (Szatmari, 2000). Within and across these diagnostic groups, there is considerable heterogeneity and variability in the expression of communication symptoms. Nevertheless, it seems likely that the *same* symptoms are found in both syndromes, suggesting much greater similarity between them than is suggested by the most widely used classification systems (APA, 1994).

Linguistic Impairments in Autism Spectrum Disorders

In contrast to the universal nature of communication deficits in autism spectrum disorders, language abilities vary on a very broad continuum. At one end, there are children with autism or Asperger syndrome whose vocabulary, grammatical knowledge, and phonologic skills are within the normal range of functioning, whereas at the other end, a significant proportion of the autistic population remains essentially nonverbal (Lord and Paul, 1997). Early psycholinguistic studies investigated these structural aspects of language by comparing verbal children with autism to other children with mental retardation or other syndromes, such as Down syndrome (e.g., Bartolucci et al., 1976; Bartolucci and Pierce, 1977; Pierce and Bartolucci, 1977; Bartolucci et al., 1980; Tager-Flusberg, 1981, 1985; Boucher,

1988; Tager-Flusberg et al., 1990). The conclusions drawn from these studies were that autism does not involve *specific* deficits in vocabulary, grammar, or phonology, because the children with autism were not different in their performance from the control groups matched on language and general cognitive ability. However, these studies included small, perhaps unrepresentative, samples of children with autism. Furthermore, they did not provide a systematic evaluation of the profile of abilities or deficits across the different domains of language, leaving much unknown about the language impairments that may be present in the majority of children with autism.

In a different set of studies, Bartak et al. (1975, 1977) compared their sample of children with autism to a group of children with specific language impairment (SLI) matched on nonverbal IQ. SLI is a disorder that is diagnosed on the basis of language levels that fall significantly below age expectations in the absence of other conditions (e.g., hearing loss, mental retardation, evidence of organic pathology). Bartak and colleagues found that the autism group was more impaired on measures of vocabulary and grammatical comprehension, but similar to the language-disordered children on measures of expressive grammar, suggesting some parallels in the kinds of language deficits found in autism and SLI.

Kjelgaard and Tager-Flusberg (2001) investigated language profiles in a large group of verbal children with autism between the ages of 5 and 14 using a battery of standardized tests tapping vocabulary knowledge, higher order receptive and expressive grammatical and semantic skills, and a nonsense word repetition test, which taps the ability to imitate wordlike strings that vary in length. Based on their performance on these language tests, the children were divided into subgroups. About one-quarter of the children had language scores that all fell within the normal range. The remaining children had language scores across these measures that were significantly below the mean. These children had distinctive profiles of performance across the language measures, suggesting that they formed a subtype of children with autism and language disorder. Although there was a moderate relationship between language scores and IQ, there were children with high and low IQ scores, and normal or delayed onset of language milestones, in both the normal and impaired language subgroups.

The profile of scores of the language-disordered children with autism resembled closely the profiles reported for children with SLI, supporting some of the findings from Bartak et al. (1975, 1977). The poor performance of these children on the nonsense word repetition test was especially significant, because this measure is considered highly sensitive to the diagnosis of SLI (cf. Gathercole and Baddeley, 1990; Bishop et al., 1996; Dollaghan and Campbell, 1998; Tager-Flusberg and Cooper, 1999). Children with SLI also have grammatical deficits, especially in marking verbs for tense (Rice and Wexler, 1996; Bedore and

Leonard, 1998). One study found that children with autism who were diagnosed as language disordered on the basis of performance on standardized tests, performed significantly worse than autistic children without language disorder on experimental tasks designed to tap children's ability to produced past tense and present tense grammatical morphology (Roberts et al., 2000). A second study found that these language-disordered children with autism omitted the past tense morpheme in spontaneous speech to the same degree as a comparison group of children with SLI (Condouris et al., 2002).

Taken together, these studies indicate that there are important parallels between the language profile of a subgroup of children with autism and what is known about the language characteristics of children with SLI. There may also be other subgroups on the autism spectrum that reflect different kinds of language disorder. For example, a significant number of children with autism never acquire language, and some of these children may be diagnosed as having verbal dyspraxia, defined as a child with severely dysfluent or very impaired speech articulation, often with little or no productive language (cf. Rapin and Allen, 1983; Lord and Paul, 1997). Little is known about these children, because there has been almost no language and communication research with autistic children who have no functional language.

How might one interpret the close parallels between autism, SLI, and other language disorders? Churchill (1972) proposed that autism was simply an extreme form of SLI, but this theory was rejected on the basis of detailed comparative studies by Bartak et al. (1975, 1977), showing that children with autism had social-communicative deficits not found in children with language disorder. Rutter (1965) proposed that autism and SLI are overlapping populations, and this view has been endorsed more recently by Bishop (2000) and Tager-Flusberg (2003). Tager-Flusberg (2003) argued that there is a subgroup of children with autism who also have SLI. On this view, SLI or other forms of language impairment (e.g., verbal dyspraxia) may be co-occurring disorders in some, but not all, children with autism. Thus, SLI or language disorder is not a defining feature of autism, because there are clearly children with autism without any linguistic deficits, but it does occur in a significantly large subgroup of the population.

Neurobiological Substrate for Language Impairment in Autism

If subgroups within autism and SLI share common language deficits, then one would hypothesize that they would show similar atypical patterns of brain structure. Thus far, there have been several studies of brain structure in SLI using

magnetic resonance imaging (MRI). The most consistent finding in these studies is that children and adults with SLI show different patterns of brain asymmetry compared with non-SLI controls. In normal individuals, left cortical regions, especially in key language areas (perisylvian region, planum temporale, and Heschel's gyrus), are enlarged relative to the size of those regions in the right hemisphere. In contrast, individuals with SLI or with language-based learning disorders show reduced or reversed asymmetries in these areas (Galaburda, 1989; Jernigan et al., 1991; Plante et al., 1991; Leonard et al., 1996; Clark and Plante, 1998).

A small number of recent studies have investigated structural brain abnormalities in autism using MRI. Herbert and colleagues (2002) compared 16 boys with autism (all with normal nonverbal IQ scores) to 15 age-, sex-, and handedness-matched normal controls. Their main findings were that the autistic boys had significant reversal of asymmetry in the inferior lateral frontal cortex, which was 27 percent larger in the right hemisphere, compared to 17 percent larger in the left hemisphere for the normal controls. There were also significant differences between the autism and control groups in the asymmetry patterns in the planum temporale. Although both groups showed a left-hemisphere asymmetry, this was more extreme in the autistic boys (25% leftward asymmetry for autism compared to only 5% in the controls).

These findings were replicated in a different sample of boys with autism in a study by De Fosse and colleagues (2002). In this study, the boys with autism were divided into those with language impairment and those without, based on standardized language test scores. A group of matched boys with SLI, as well as normal controls, were also included in this study. De Fosse et al. found that abnormal asymmetry patterns (right asymmetry in inferior frontal cortex; more extreme left asymmetry in the planum temporale) were found for the autistic boys with language impairment and the SLI group; however, the autistic boys with normal language had the same asymmetry patterns as the control children. The findings for the planum temporale were not replicated in a study comparing adults with autism and age-matched normal controls (Rojas et al., 2002). Rojas and colleagues found that their autistic adults had significantly reduced left hemisphere planum temporale volumes, and no hemispheric asymmetry in this important language region. Perhaps methodologic differences can explain these conflicting findings. Rojas et al. (2002) studied adults rather than children and included women in their sample; their groups were not matched for IQ. Further studies are clearly needed to explore the structural abnormalities in brain regions subserving language in both children and adults with autism.

Conclusion

Since Kanner (1943) first described autism, much has been learned about the language and communication abnormalities that define this heterogeneous syndrome. Across the spectrum of autism disorders, there are universal and specific deficits in vocal expression and social communication. These deficits include many different features and may be expressed in a variety of ways among individuals with autism or Asperger syndrome. Linguistic deficits are not universal in autism. Instead, it seems likely that there are different subgroups: some children have no structural language impairments whereas others have co-occurring language disorders, with SLI quite prevalent among verbal children with autism. These findings have important implications for how children with autism are diagnosed and evaluated in the domain of language and the kinds of interventions they will need. Future research should address variations in the development of the brain and cognitive mechanisms that underlie the broad range of language and communicative impairments in autism.

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5

Memory and Executive Functions in Autism

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The neurobiologic bases of the cognitive changes associated with autism are not well understood. The ultimate goal is to fully characterize the nature and extent of changes in all domains of behavior, including the processing of language, social skills, and all aspects of cognitive function. It is beyond the scope of this chapter to deal with all these aspects of autistic behavior. With regard to cognitive function, the symptoms of autism appear at a time when many skills are normally being acquired or refined and are thought to reflect the consequences of abnormalities in the early development of the central nervous system. Specifically, the temporal lobe and prefrontal cortex and their roles in memory and executive function, respectively, are coming under close examination. This chapter focuses on these two emerging areas of cognitive function.

The neural system or systems underlying memory function are responsible for receiving, storing, and making available vast quantities of diverse information. However, the exact processes through which this function is accomplished are not fully understood. There is no consensus among cognitive psychologists and neurobiologists as to how many different neural systems subserve memory or, within each system, how many stages are involved. However, there is general agreement that memory is not a unitary phenomenon.

The neural system or systems underlying the executive functions are responsible for the broad skills of organization, regulation, and awareness. Organizational skills include the abilities to concentrate, make decisions, plan, and sequence. Regulational skills include the abilities to initiate behavior, repeat responses, and control temper. Awareness skills include the abilities to recognize deficits in oneself, unintentionally comply to the social norms, and use feedback to regulate behavior. As is true for the memory system, there is no consensus among cognitive psychologists and neurobiologists as to how may different neural systems or networks subserve the executive functions.

Overall, the population of individuals with autism represents a heterogeneous group in terms of the severity of impairment, the overt behavioral profile, and the presence of any "special talents." According to the DSM-IV-TR (APA, 2000), the symptoms of autism are characterized by impairments of three types. First, there is an impairment of reciprocal social interaction, which is illustrated by a lack of awareness of the existence of others. Second, there is an impairment of communication, which includes both verbal dysfunctions, such as a lack of speech or echolalia, and impairment of nonverbal communication, such as facial gestures. Third, there is an impairment of the behavioral repertoire of an autistic child, characterized by repetitive perseverations, such as stereotyped body movement. In addition to the criteria listed in the DSM-IV-TR, Rutter (1978) included an insistence on sameness.

It has been speculated that at least some of the symptomatology of autism could be explained in terms of a memory deficit. For example, off-topic comments made by an autistic individual may occur simply because he or she cannot recall the topic of the conversation. Similarly, the repetitive behaviors could be explained by an inability to recall previously performing an activity. Investigators comparing the performance of autistic children to adult patients with amnesia due to either Korsakoff disease or medial temporal lobe damage (hippocampus) have found the following. Autistic children, like amnesic patients, display an intact immediate memory, relatively good performance on tests of visual spatial reasoning (Bartak et al., 1975), preserved capacity to learn and retain motor skills (Goldstein, 1959; Hermelin and O'Conner, 1970), and an impairment of recent memory, as evidenced by their inability to verbally report activities in which they have participated (Boucher, 1981). However, the impairment of recent memory found in autistic children appears to be relatively specific to verbal material, whereas that in amnesic adults is more global. Autistic children are impaired at recalling auditory-verbal material following a delay (Boucher and Warrington, 1976), yet are unimpaired at recent memory for static visual stimuli (Selfe, 1983). Similarly, even though autistic children display an intact immediate memory, their span for words differs more from their span for digits than is the case in other groups of children (Boucher, 1978).

At least two possibilities exist that account for the discrepancies found in the behavioral profiles of autistic children and adult amnesic patients. First, the adult who develops amnesia has a vast store of information from which to draw, including a working understanding of language. However, if there is a memory impairment associated with autism, then the child developing autism by 30 months of age would presumably be impaired at learning all new information, including language. Second, the etiology of the two disorders may be completely different. Unfortunately, little is known about the etiology of autism at this

time. Autopsy reports on autistic individuals have shown bilaterally symmetrical abnormalities confined primarily to the hippocampal complex and closely associated areas, as well as to the cerebellum (Bauman and Kemper, 1985, 1986, 1987). Hippocampal damage of this type would be consistent with an impairment of recent memory. However, because of the extreme heterogeneity of symptoms among the autistic population, further studies are needed to determine if these findings are representative of the entire autistic population.

Bachevalier (1991) suggested an animal model for childhood autism, based on work with infant monkeys that have sustained damage to the limbic cortices thought to subserve recent memory functions. She has found that infant monkeys with combined bilateral neonatal ablations of the amygdala, hippocampus, and overlying cortices display severe memory deficits (Bachevalier and Mishkin, 1988), which are accompanied by socioemotional abnormalities that are similar to those seen in autistic children (Merjanian et al., 1986). Interestingly, subsequent work has failed to demonstrate a memory deficit in infant monkeys following bilateral neonatal ablations limited to the hippocampus and overlying cortices (Bachevalier and Mishkin, 1991). Thus, even the proposed animal model of autism does not give us a clear indication of the presence or absence of a memory deficit in autism.

It is also plausible that what is being seen as an impairment of memory function with autism is actually an impairment in information processing and organization, leading to impaired performance on memory tests. For example, when one study (Minshew and Goldstein, 1993) looked at the performance of autistic subjects by using the California Verbal Learning Test (a test of verbal list learning and recall), and performance was broken down into subscores, an interesting profile began to emerge. On 27 of 33 variables assessed, it was not possible to discriminate between the autistic subjects and the control subjects, suggesting that there is no clear memory disorder associated with autism. However, the group mean performance of the autistic subjects was lower than that of the control subjects on 30 of 33 variables, suggesting that the autistic subjects were less efficient in their performance on this test. In a follow-up study, Minshew and Goldstein (2001) assessed the performance of autistic subjects on a variety of memory and learning tests. They found that the impairments seen were induced by a failure to initiate organizing strategies and a sensitivity to the complexity of the material, adding further support to the notion that information processing, rather than memory per se, is impaired.

However, it seems that there is a relatively clear impairment of executive function in autism. The repetitive behaviors seen in autism suggest a deficit in the collection of regulational skills, whereas the impairment of reciprocal social interaction suggests an impairment of awareness skills. Indeed, some investiga-

tors have gone as far as to suggest the "executive function hypothesis" of autism (Pennington, 1994; Ozonoff, 1995), which promotes the notion that impairments of executive function underlie many of the symptoms of autism.

One classic test of executive function is the Wisconsin Card Sorting Test. This test was first developed by Berg (1948) as a measure of abstraction and cognitive flexibility. The test was further developed into a clinical tool by Heaton and colleagues (Heaton, 1981; Heaton et al., 1993). When this test was administered to high-functioning verbal adults with autism, an impairment was found relative to a matched control sample (Rumsey, 1985). When a finer-grained analysis was done on the performance of the autistic subjects, difficulties were seen in overall problem solving. However, no difference was seen between the autistic subjects and control subjects in the number of perseverative errors (Rumsey and Hamburger, 1988). When a modified version was administered to lower-functioning autistic children, it was found that the autistic group made more errors and perseverative responses than did controls (Prior and Hoffmann, 1990).

Deficits in performance by autistic subjects have also been described on a variety of other tests of executive function. On the Tower of Hanoi and Tower of London tests of problem solving, the performance of autistic subjects was found to be impaired with respect to the performance of control subjects (Rumsey and Hamburger, 1988; Ozonoff et al., 1991; Hughes et al., 1994; Ozonoff and McEvoy, 1994). On the Trailmaking Test, a test of sequencing and cognitive flexibility, the performance scores of autistic subjects were found to be below those of control subjects (Rumsey and Hamburger, 1988). And on the Spatial Reversal and Go-No-Go tests, classic tests of frontal lobe function that have been used with nonhuman primates, the performance of autistic subjects was also shown to be impaired (McEvoy et al., 1993; Ozonoff et al., 1994).

As investigations into the cognitive abilities of autistic individuals continue, it is important to take a close look at the demands of the tests being used. As described above, several investigators found impairments in autistic subjects with the Wisconsin Card Sorting Test. This suggests that autism results in impairments of abstraction and cognitive flexibility. However, when a computerized version of this test was given, the differences between the autistic and control subjects were attenuated (Ozonoff, 1995). It is quite plausible that impairments in social interactions in the autistic subjects have a synergistic effect on performance. When the test requirements for social interactions are reduced, performance is enhanced.

Clearly, more work is needed in the development of behavioral instruments to assess memory and executive function, as well as the other cognitive domains, in childhood autism and autism-related disorders. A finer-grained analysis of acquisition and retention of stimulus-stimulus, and stimulus-response relations,

together with the further delineation of patterns and types of neuropathologic changes and development of animal models in autism (Bachevalier, 1991) will significantly shorten the path leading to the understanding of the neurobiologic basis of this disorder.

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6

The Vagus: A Mediator of Behavioral and Physiologic Features Associated with Autism

Stephen W. Porges, Ph.D.

The vagus nerve, as a system, provides a rich organizing principle to investigate several of the behavioral, psychological, and physiologic features associated with a diagnosis of autism. The vagus is not only a cranial nerve meandering through the periphery, but also an important bidirectional conduit carrying specialized motor and sensory signals that are part of a larger integrated feedback system that includes brain structures involved in the regulation of visceral state and affect. The premise of this chapter is that several features of autism become more understandable if a more integrated model of the nervous system is applied in which the vagus is a critical component.

The Vagus and Affect Regulation

The relation between affect and vagal afferent activity is not a recent idea. Darwin (1872) noted in *The Expression of Emotions in Man and Animals* the importance of the bidirectional neural communication between the heart and the brain via the "pneumogastric" nerve, now known as the vagus nerve.

For Darwin, emotional state represented a covariation between facial expression and autonomic tone. However, he did not elucidate the specific neurophysiologic mechanisms. Our current knowledge of the neuroanatomy, embryology, and phylogeny of the nervous system was not available to Darwin. In Darwin's time, it was not known that vagal fibers originated in several medullary nuclei, that branches of the vagus exerted control over the periphery through different feedback systems, that sensory information conveyed through the vagus regulated structures in the brain, and that the function of the branches of the vagus followed a phylogenetic principle.

Current research emphasizes the importance of the vagal afferents in the regulation of visceral state, mood, and affect. Studies have demonstrated that stim-

ulation of vagal afferents regulate brain structures involved in epilepsy (Boon et al., 2001), depression (George et al., 2000), and even repetitive self-destructive behaviors often associated with autism (Murphy et al., 2000).

Polyvagal Theory: Three Neural Circuits Regulating Reactivity

Evolutionary forces have molded both human physiology and behavior. Via evolutionary processes, the mammalian nervous system has emerged with specific neural and behavioral features that react to challenge in order to maintain visceral homeostasis. These reactions change physiologic state and, in mammals, limit sensory awareness, motor behaviors, and cognitive activity. To survive, mammals must determine friend from foe, evaluate whether the environment is safe, and communicate with their social unit. These survival-related behaviors are associated with specific neurobehavioral states that limit the extent to which a mammal can be physically approached and whether the mammal can communicate or establish new coalitions. Thus, environmental context can influence neurobehavioral state, and neurobehavioral state can limit a mammal's ability to deal with the environmental challenge.

Through stages of phylogeny, mammals, and especially primates, have evolved a functional neural organization that regulates visceral state to support social behavior. The polyvagal theory (Porges, 1995, 1997, 1998, 2001) emphasizes the phylogenetic origins of brain structures that regulate social and defensive behaviors, domains compromised in individuals with autism. The polyvagal theory proposes that the evolution of the mammalian autonomic nervous system provides the neurophysiologic substrates for the emotional experiences and affective processes that are major components of social behavior. The theory proposes that physiologic state limits the range of behavior and psychological experience. In this context, the evolution of the nervous system determines the range of emotional expression, quality of communication, and the ability to regulate bodily and behavioral state. The polyvagal theory links the evolution of the autonomic nervous system to affective experience, emotional expression, facial gestures, vocal communication, and contingent social behavior. Thus, the theory provides a plausible explanation of several social, emotional, and communication behaviors and disorders associated with autism.

The polyvagal construct was introduced to emphasize and document the neurophysiologic and neuroanatomical distinction between two branches of the vagus and to propose that each vagal branch is associated with a different adaptive behavioral strategy. The theory proposes that the different branches are related to unique, adaptive behavioral strategies and articulates three phylogenetic stages of the development of the mammalian autonomic nervous sys-

tem. These stages reflect the emergence of three distinct subsystems, which are phylogenetically ordered and behaviorally linked to social communication (e.g., facial expression, vocalization, listening), mobilization (e.g., fight-flight behaviors), and immobilization (e.g., feigning death, vasovagal syncope, and behavioral shutdown). The mobilization system is dependent on the functioning of the sympathetic nervous system. The most phylogenetically primitive component, the immobilization system, is dependent on the unmyelinated or "vegetative" vagus, which is shared with most vertebrates. With increased neural complexity due to phylogenetic development, the organism's behavioral and affective repertoire is enriched (see Table 6.1).

The theory emphasizes the functional aspect of neural control of both the striated muscles of the face and the smooth muscles of the viscera, because their functions rely on common brainstem structures. It does not make any assumptions regarding structural damage to either the vagal systems or the brain structures that regulate brainstem structures associated with the vagal systems. Thus, although the compromised brainstem features described by Rodier and colleagues (1996) are consistent with the predictions of the polyvagal theory, the theory emphasizes functional deficits and does not necessarily assume structural damage.

By investigating the phylogeny of the regulation of the vertebrate heart (Morris and Nilsson, 1994), three principles can be extracted. First, there is a phylogenetic shift in the regulation of the heart from endocrine communication, to unmyelinated nerves, and finally to myelinated nerves. Second, there is

TABLE 6.1. The Three Phylogenetic Stages of the Neural Control of the Heart Proposed by the Polyvagal Theory

Phylogenetic Stage	*ANS Component*	*Behavioral Function*	*Lower Motor Neurons*
III	Myelinated vagus	Social communication, self-soothing and calming, inhibit sympathetic-adrenal influences	Nucleus ambiguus
II	Sympathetic-adrenal	Mobilization (active avoidance)	Spinal cord
I	Unmyelinated vagus	Immobilization (death feigning, passive avoidance)	Dorsal motor nucleus of the vagus

a development of opposing neural mechanisms of excitation and inhibition to provide rapid regulation of graded metabolic output. Third, with increased cortical development, the cortex exhibits greater control over the brainstem via direct (e.g., corticobulbar) and indirect (e.g., corticoreticular) neural pathways originating in motor cortex and terminating in the source nuclei of the myelinated motor nerves emerging from the brainstem (e.g., specific neural pathways embedded within cranial nerves V, VII, IX, X, and XI), controlling visceromotor structures (i.e., heart, bronchi, thymus) and somatomotor structures (muscles of the face and head).

These phylogenetic principles provide a basis for speculations regarding the behavioral and physiologic responses associated with autism. With this new vagal system, transitory incursions into the environment or withdrawals from a potential predator can be initiated without the severe biologic cost associated with sympathetic-adrenal activation. Paralleling this change in neural control of the heart is an enhanced neural control of the face, larynx, and pharynx that enables complex facial gestures and vocalizations associated with social communication. This phylogenetic course results in greater central nervous system regulation of physiologic state that supports behaviors needed to engage and disengage with environmental challenges.

The Vagal Brake

Due to the tonic vagal influences to the sinoatrial node (i.e., the heart's pacemaker), resting heart rate is substantially lower than the intrinsic rate of the pacemaker. When the vagal tone to the pacemaker is high, the vagus acts as a brake on the rate at which the heart is beating (Porges et al., 1996). When vagal tone to the pacemaker is low, there is little or no inhibition of the pacemaker. Thus, neurophysiologically, the vagal brake provides a mechanism to rapidly switch between physiologic states that either support social communication or mobilization. Functionally, the vagal brake, by modulating visceral state, enables the individual to rapidly engage and disengage with objects and other individuals and to promote self-soothing behaviors and calm behavioral states. These behaviors are obviously compromised in autism. Is it possible that autism is associated with a deficit in the vagal brake and an inability to switch between neurobiologic states that foster either defensive or social behaviors?

The Social Engagement System

The polyvagal theory provides an explicit neurobiologic model of how difficulties in spontaneous social behavior are linked to both facial expressivity and the

regulation of visceral state. The theory proposes a possible mechanism to explain how these difficulties might form a core domain of several psychiatric profiles. Relevant to autism are the specific deficits in both the somatomotor (e.g., poor gaze, low facial affect, lack of prosody, difficulties in mastication) and visceromotor (e.g., difficulties in autonomic regulation resulting in cardiopulmonary and digestive problems) areas of the social engagement system. Deficits in the social engagement system compromise spontaneous social behavior, social awareness, affect expressivity, prosody, and language development. In contrast, interventions that improve the neural regulation of the social engagement system, hypothetically should enhance spontaneous social behavior and state and affect regulation, reduce stereotypical behaviors, and improve language skills.

During the development of the human embryo, components of several cranial nerves, known as special visceral efferent pathways, develop together to form the neural substrate of a social engagement system (see Porges, 1998). This system, as illustrated in Figure 6.1, provides the neural structures involved in social and emotional behaviors. The social engagement system has a control component in the cortex (i.e., upper motor neurons) that regulates brainstem nuclei (i.e., lower motor neurons) to control eyelid opening (e.g., looking), facial muscles (e.g., emotional expression), middle ear muscles (e.g., extracting the human voice from background noise), muscles of mastication (e.g., ingestion), laryngeal and pharyngeal muscles (e.g., vocalization and language), and head turning muscles (e.g., social gesture, orientation). Collectively, these muscles function as filters that limit social stimuli (e.g., observing facial features, listening to the human voice) and determinants of engagement with the social environment. The neural control of these muscles determines social experiences. In addition, the source nuclei (i.e., lower motor neurons) of these nerves, which are located in the brainstem, communicate directly with an inhibitory neural system that slows heart rate, lowers blood pressure, and actively reduces arousal to promote calm states consistent with the metabolic demands of growth and restoration of human neurophysiologic systems.

Direct corticobulbar pathways reflect the influence of frontal areas of the cortex (i.e., upper motor neurons) on the regulation of this system. Moreover, afferent feedback through the vagus to medullary areas (e.g., the nucleus of the solitary tract, which is the source nucleus of the afferent vagus) influences forebrain areas that are assumed to be involved in several psychiatric disorders. In addition, the anatomical structures involved in the social engagement system have neurophysiologic interactions with the hypothalamic-pituitary-adrenal (HPA) axis, the neuropeptides of oxytocin and vasopressin, and the immune system (Porges, 2001). As a cluster, the difficulties with gaze, extraction of the human voice, facial expression, head gesture, prosody, and state regulation are

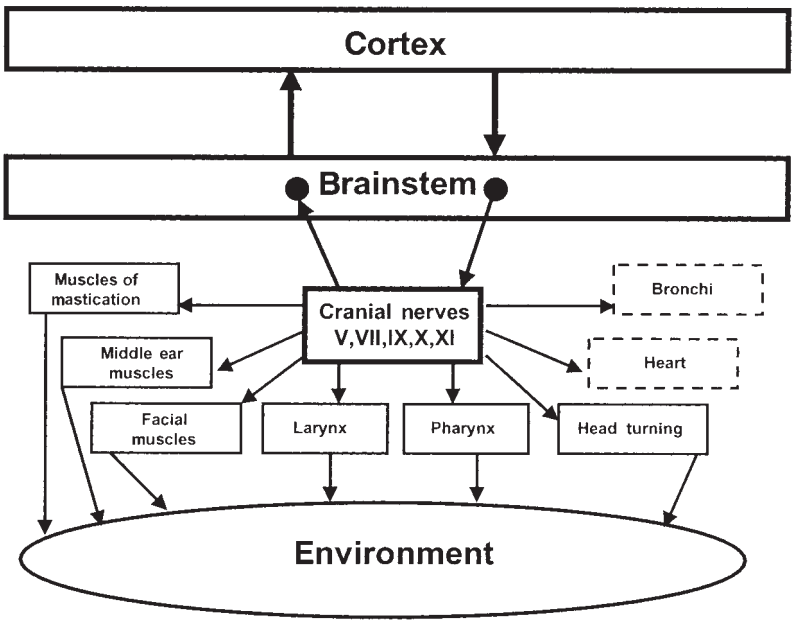

FIGURE 6.1. The social engagement system. Social communication is determined by the cortical regulation of medullary nuclei via corticobulbar pathways. The social engagement system consists of a somatomotor component (special visceral efferent pathways that regulate the muscles of the head and face) and a visceromotor component (the myelinated vagus that regulates the heart and bronchi). Solid blocks indicate the somatomotor component. Dashed blocks indicate the visceromotor component.

common features of individuals with autism. For example, the neural pathway that raises the eyelids also tenses the stapedius muscle in the middle ear, which facilitates hearing the human voice (Borg and Counter, 1989). Thus, the neural mechanisms for making eye contact are shared with those needed to listen to the human voice.

Studies have demonstrated that the neural regulation of middle ear muscles, a necessary mechanism to extract human voice from loud low frequency background noise, is defective in individuals with language delays, learning disabilities, and autistic spectrum disorders (Thomas et al., 1985; Smith et al., 1988). Middle ear infection (i.e., otitis media) may result in a total inability to elicit the "reflexive" contraction of the stapedius muscles (Yagi and Nakatani, 1987). Disorders that influence the neural function of the facial nerve (i.e., Bell's palsy) not only influence the stapedius reflex (Ardic et al., 1997) but also affect the patient's ability to discriminate speech (Wormald et al., 1995). Thus, the observed

difficulties that many autistic individuals have in extracting the human voice from background sounds may be dependent on the same neural system that is involved in facial expression.

Predictions Based on Polyvagal Theory

Observations of the behaviors and physiologic response of autistic individuals suggest that they have great difficulties in recruiting the neural circuit that regulates the social engagement system. It appears that autism is associated with autonomic states that remove the individual from direct social contact by supporting the adaptive defensive strategies of mobilization (i.e., fight-flight behaviors) or immobilization (i.e., shut-down). Behaviorally, the retraction of the neural regulation of the social engagement system is expressed as limited use and regulation of the muscles of the face and head. The functional consequences limit facial expressions and head gestures, compromise the ability to extract the human voice from background sounds, and reduce prosody.

Neurophysiologically, because the vagus is integrated into several feedback systems involving both peripheral and central structures, depression or dysregulation of the vagus might be manifested on several levels. First, it may compromise the regulation of visceral organs, such as the gut, heart, and pancreas. Second, because the vagus is involved in the modulation of pain and the regulation of cytokines and the HPA axis, there may be regulational disorders in those systems. Third, because the brainstem areas regulating the myelinated vagal system provide both output and input to feedback systems involving other brain structures, the vagal system may provide a portal to assess and stimulate higher neural processes. Although there is a limited scientific literature evaluating the role of vagus in autism, the plausibility of these predictions will be reviewed in this chapter and discussed against the current literature, which includes studies with other clinical populations and animal preparations.

Vagal Regulation of Heart Rate and Heart Rate Variability

Because vagal efferent pathways to the heart are cardioinhibitory, changes in vagal tone can influence the metrics used to monitor heart rate and heart rate variability. In general, greater cardiac vagal tone produces slower heart rate and regulates the transitory changes in heart rate in response to stimulation. The myelinated vagal efferents that synapse on the sinoatrial node have a respiratory rhythm. This rhythmic increase and decrease in cardioinhibitory activity through the vagus produces a cardiac rate rhythm known as respiratory sinus arrhythmia. The greater the cardioinhibitory influence through the vagus, the

greater the rhythmic increases and decreases in this heart rate pattern. Thus, the amplitude of respiratory sinus arrhythmia provides a sensitive index of the influence the myelinated vagus has on the heart. The rapid changes in heart rate in response to specific stimuli are primarily under vagal control. The characteristic heart rate pattern to stimulus changes—an immediate deceleration followed by either a continued deceleration or an acceleration—is primarily due to dynamic increases or decreases in cardioinhibitory activity through the myelinated vagus. The literature suggests that autism is associated with reliable differences in the amplitude of respiratory sinus arrhythmia and the transitory heart rate response pattern to various stimuli and task demands.

An early publication by Hutt et al. (1975) reported that normal children suppressed respiratory sinus arrhythmia more than autistic children did. Similarly, Althaus et al. (1999) found that children with a pervasive developmental disorder not otherwise specified (PDD-NOS) did not suppress respiratory sinus arrhythmia. Consistent with these findings, an early study of children diagnosed with schizophrenia (Piggott et al., 1973) identified significant differences in respiration and in the covariation between respiration and heart rate. The schizophrenic children had significantly faster and more shallow breathing patterns, a pattern consistent with reduced vagal efferent activity.

Other studies report that autistic children have dampened transitory heart rate responses to a variety of stimulation. Zahn, Rumsey, and Van Kammen (1987) reported unusually small deceleratory heart rate responses to auditory stimulation. Palkovitz and Wiesenfeld (1980) reported dampened heart rate responses to socially relevant speech, nonsense phrases, and a 500 Hz tone. Corona et al. (1998) reported that the heart rate of children with autism did not change across conditions.

Vagal Nerve Stimulation

Although not currently being used to treat autism, vagal nerve stimulation has been effective in treating epilepsy and depression. Vagal nerve stimulation is based on the assumption that stimulation of vagal afferents has a direct effect on the regulation of higher brain structures. The source nucleus of the vagal afferents is the nucleus of the solitary tract. This medullary nucleus plays an important role in the regulation of behavioral state, respiration, and blood pressure, and in conveying information to higher brain structures. The nucleus of the solitary tract relays the incoming sensory information via three primary pathways: (1) feedback to regulate the periphery, (2) direct projections to the reticular formation in the medulla, and (3) ascending projections to the forebrain, primarily through the parabrachial nucleus and the locus ceruleus. The parabrachial

nucleus and the locus ceruleus send direct connections to all levels of the forebrain (e.g., hypothalamus, amygdala, the thalamic regions that control the insula and orbitofrontal and prefrontal cortices), areas that have been implicated in neuropsychiatric disorders. Thus, vagal afferent stimulation has direct input to both the lower motor neurons in the brainstem and the upper motor neurons in the cortex that regulate the social engagement system. Recent reviews provide a detailed description of the neurophysiologic basis for the intervention (George et al., 2000) and provide an explanation of the neural mechanisms involved in treating depression with vagal nerve stimulation (Marangell et al., 2002). Missing from these explanations is an acknowledgment of the communication between vagal afferents and the source nuclei of the nerves that regulate striated muscles of the face and head (i.e., special visceral efferent pathways), which collectively form the motor part of the social engagement system. It is this interaction that is emphasized in the polyvagal theory (Porges, 2001).

Extrapolating from the vagal nerve stimulation model, one might speculate that other forms of vagal stimulation might have beneficial effects. Behaviorally, one of the most potent strategies for vagal stimulation is to stimulate the peripheral baroreceptors that regulate blood pressure. Rocking and swinging, in which the position of the head is changed relative to the position of the heart, will stimulate the baroreceptors and engage this feedback loop. This suggests that the frequently observed rocking and swinging behaviors in autistic individuals may reflect a naturally occurring biobehavioral strategy to stimulate and regulate a vagal system that is not efficiently functioning.

One publication reported that vagal nerve stimulation reduced autistic-like behaviors (Murphy et al., 2000). In this study, vagal stimulation was administered to six patients with hypothalamic hamartoma, a congenital brain malformation that is associated with medically refractory epilepsy and injurious autistic behavior. Four of the six patients had autistic behaviors that included poor communication, ritualisms, compulsions, no social skills, and injury to self and others. The authors report that during vagal nerve stimulation, all four showed impressive improvements in behavior. In one subject, the behavioral improvements were immediately reversed when the vagal nerve stimulation was temporarily discontinued without worsening of seizure frequency.

Vagal Regulation of the Gut

Due to the high prevalence of gastrointestinal symptoms in individuals with autism (Horvath and Perman, 2002; Wakefield et al., 2002), there has been an interest in a possible link between gut and brain as a determinant of autism. This interest was stimulated by reports from parents who indicated that the adminis-

tration of intravenous secretin reduced autistic symptoms. However, there has been no evidence for the efficacy of secretin when it was administered in a randomized, placebo-controlled, double-blind clinical trial (Owley et al., 2001).

Current research suggests that the prevalence of gastrointestinal symptoms represents an unsolved problem in autism. However, if we conceptualize the problem from a "vagal" perspective, we can identify the vagus as a primary regulator of the gut, with vagal afferents providing important information to brain structures. Support for this argument comes from animal studies in which it has been demonstrated that the vagus is involved in the regulation of secretin (Lu and Owyang, 1995; Li, Chang, and Chey, 1998). Thus, given the compromised behavioral components of the social engagement system, it is not surprising to find that the vagal regulation of gastrointestinal processes is also compromised in autistic individuals.

Additional information regarding the role that vagal afferents from the gut have in modulating sensory experiences comes from research on eating disorders. Research suggests that vagal afferents are involved not only in regulating satiety via vasovagal reflexes but also in regulating nociceptive sensations via solitary-spinal pathways. Faris et al. (2000) and Raymond et al. (1999a) have proposed that vagal afferent activation by binge-eating and vomiting also activates the descending pain inhibitory pathway resulting in an elevated pain threshold. Similarly they have reported elevated pain thresholds in anorexia nervosa subjects (Raymond et al., 1999b). Their research has led to administering ondansetron as an intervention for bulimia nervosa (Faris et al., 2000). Ondansetron is marketed for the prevention of vagally mediated emesis caused by cancer chemotherapeutic agents.

The Vagus and the Immune System

The subdiaphragmatic vagal afferents may be conceptualized as providing a targeted signal to the central structures that regulate immune function. Other researchers have linked the vagal efferent pathways to immune function. Bulloch and Pomerantz (1984) described motor pathways via the vagus to the thymus. The link between the vagal regulation of immune function and the polyvagal theory is not clear. However, it might be plausible to speculate that the neural mediation of the myelinated vagus may, via direct influence on thymus and direct inhibition of the sympathetic nervous system, trigger a physiologic state that would promote immune function. Likewise, mobilization strategies, resulting in a withdrawal of vagal tone to the heart, increased sympathetic tone, and the release of cortisol, have been associated with suppressed immune function. More relevant to the expression of psychiatric disturbances is the find-

ing that the afferent vagus mediates behavioral depression, but not fever, in response to peripheral immune signals following abdominal inflammation (Konsman et al., 2000). Consistent with this model, it has been reported that autism spectrum disorder patients with developmental regression express excessive innate immune responses (Jyonouchi, Sun, and Le, 2001).

Vagal Regulation of the HPA Axis

The vagus is involved in the regulation of the HPA axis. Vagal afferents exhibit an inhibitory influence on HPA axis and reduce cortisol secretion (e.g., Bueno et al., 1989; Miao et al., 1997). Studies (Cacioppo et al., 1995; Gunnar et al., 1995) have demonstrated a covariation between increases in cortisol and decreases in cardiac vagal tone (i.e., the amplitude of respiratory sinus arrhythmia). Thus, there appears to be a coordinated response that functions to promote metabolic activity and mobilization behaviors by withdrawing the vagal tone through the myelinated vagus and increasing both sympathetic activity and activation of the HPA axis.

Several studies have reported that the regulation of the HPA axis is dysfunctional in autistic children. Poorly developing autistic children were more likely to have an abnormal diurnal rhythm and an abnormal response on the dexamethasone suppression test than less severe cases (Hoshino et al., 1987). The results suggest that the negative feedback mechanism of the HPA axis may be disturbed in autistic children, especially in poorly developing individuals. Similarly, Jensen et al. (1985) reported that most of the autistic patients studied failed to suppress cortisol with the dexamethasone test. Consistent with these reports, Jansen et al. (2000) reported the PDD-NOS children had a diminished cortisol response to physical stress.

The Vagal System as an Organizing Principle

In this chapter, I have illustrated how the vagus is involved in the expression of several disparate symptoms associated with autism. Consistent with the polyvagal theory, the symptom clusters are associated with components of the vagal system. First, there are the behavioral characteristics linked to the neural regulation of the striated muscles of the face via special visceral efferent pathways (i.e., the somatomotor component of the social engagement system). Second, autism is associated with dysfunctional regulation of target organs (e.g., heart, gut) regulated by vagal efferent pathways (i.e., the visceromotor component of the social engagement system). Third, the vagal afferents exert a powerful regulatory influence on several systems—including visceral and tactile pain thresholds, the HPA

axis, and the immune system—that are dysfunctional in autism. Fourth, the nucleus of the solitary tract (the source nucleus of the afferent vagus) influences areas of the forebrain that have been speculated to be compromised in autism.

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7

Approaches to Psychopharmacology

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Autism is a neurologic disorder characterized by a disturbance in reciprocal social interaction, abnormalities in verbal and nonverbal communication, and a markedly restricted repertoire of activities and interests. In addition, cognitive delays and seizure disorders frequently co-occur in individuals with autism. Although not a part of the core diagnostic symptoms, the presence of aggression toward self and/or others, hyperactivity, impulsivity, attention deficits, hyperarousal and activation, anxiety and obsessive-compulsive symptoms, and repetitive stereotypic behaviors are frequently associated with autism.

Autism is a multidimensional disorder, and its presentation varies among individuals with respect to severity, associated behavioral symptoms, associated cognitive deficits, and prognosis. Autism is an etiologically heterogeneous disorder in which individuals with the same phenocopy (behavioral presentation) may have distinctly different neurologic substrates (brains). To date, there exists no biologic marker for the diagnosis of autism, and no specific tests can distinguish accurately and consistently among autistic individuals. Thus, medication response and side effects may vary widely among individuals.

Autistic individuals have been clinically treated with almost all classes of psychoactive medications, including antipsychotics (typical and atypical), stimulants, alpha adrenergic agents (clonidine and guanfacine), beta-blockers, fenfluramine, opioid antagonists (naltrexone), antidepressants (selective serotonin reuptake inhibitors and heterocyclic antidepressants), mood stabilizers (lithium), anticonvulsants, buspirone, and pyrodoxine/magnesium. Agents without apparent core central nervous system (CNS) effects have also been tried, such as intravenous immunoglobulin and porcine secretin.

Because there exists no "cure" for autism, medications are typically used as an adjunct to a comprehensive psychoeducational and family treatment plan and are targeted at reducing associated neuropsychiatric symptoms. Medication, in the context of a comprehensive individualized treatment program, may enhance

the child's ability to benefit from educational, behavioral, and social remediation and rehabilitative efforts.

Approaches to Medication Treatment

A review of the existing literature suggests that there are three types of approach to the use of pharmacologic interventions in autism: (1) a diagnostic approach, (2) a target-symptom approach, and (3) a scientific method approach.

The diagnostic approach to the use of medications is simply based on the presence of a diagnosis of autism or an autistic spectrum disorder alone, regardless of symptom presentation. An example of this approach might be embodied in the statement "atypical antipsychotics treat autism." The positive aspect of this approach is that this statement may be true. The negative side of this type of thinking is that atypical antipsychotics do not help all autistic individuals or remediate all symptoms of autism. Thus, the diagnostic approach may attempt to treat *all* persons with autism with the same intervention. Individual variations in symptom presentation or underlying etiology are largely discounted. Because the underlying etiology of autism is probably very heterogeneous (i.e., multiple CNS deficits may lead to a final common pathway that resembles autistic behavior), diagnostic treatment with a single agent is not realistic.

The target-symptom approach to the use of medication seeks to identify specific "target symptom(s)," irrespective of psychiatric diagnosis. This identification of target symptoms focuses the treatment on a specific aspect of the overall clinical picture. By treating these target symptoms, it is hoped that the overall burden of disease will be reduced and daily functioning improved. The most common symptoms in autism that have been "targets" of pharmacologic interventions have included hyperactivity, inattention, impulsivity, repetitive behavior/obsessive-compulsive symptoms, anxiety, and aggression toward self or others. A variation on this approach is based on the assessment and identification of comorbid psychiatric disorders. The presence of a comorbid psychiatric disorder can then be used to suggest a medication intervention. In this population, these diagnoses are made by inference based on observed behavioral symptoms. Examples of the target-symptom approach include (1) treatment of hyperactive/impulsive behavior with stimulants, (2) treatment of repetitive stereotypic behavior (compulsions) with selective serotonin reuptake inhibitor (SSRI), (3) treating the presence of comorbid depression with SSRIs, and (4) treatment of comorbid mania with a mood stabilizer.

The positive aspect of this approach is that it allows for a more specific and individualized intervention. However, there exist problems with this type of medical treatment in autism. First, there is no specific medication for the treat-

ment of autism, and a response to a particular medication does not confirm the presence of a comorbid diagnosis. Second, because many persons with autism have multiple associated symptoms that may be targets for adjunctive medication treatment, there exists a real danger of polypharmacy in these vulnerable individuals. Finally, most, if not all, of the original studies of efficacy in pediatric psychopharmacology clinical trials typically exclude individuals with autism and pervasive developmental disorder (PDD). Thus, we possess very limited data on pediatric psychopharmacology in the treatment of autistic children and adolescents with these target symptoms and comorbid disorders to help guide clinical practice.

As scientific knowledge about brain functioning in persons with autism accumulates, it is hoped that the approach to medication use will be increasingly informed by developmental neuroscience, neurobiology, genetics, structural and functional neuroimaging, neurocognition, and neuropharmacology. The scientific method approach focuses on results of investigations in these domains. By defining a specific underlying mechanism of action for symptom development and attempting to directly affect that underlying mechanism pharmacologically, a more rational approach to medication treatment can be realized. The downside of this approach is that interventions are currently curtailed by the limited extent of our present developmental neurobiologic knowledge base. Thus, the scientific method to medication treatment in persons with autism at this time represents more of an ideal hope than a reality.

Principles of Treatment

Children, adolescents, and adults with autism and their families actively struggle with symptoms that interfere with development and functioning and erode the quality of their lives. The decision to treat in the context of an evolving science and developmental neurobiologic knowledge base rests on the capacity to reduce suffering while causing no further harm. Based on existing experience, it is clear that there is an active role for medications in the treatment of persons with autism. Successful medication treatment should not only reduce psychiatric symptoms, but also enhance the individual's quality of life.

When adjunctive medication trials for associated psychiatric symptoms in persons with autism are instituted, they should be done so systematically, and an evidence-based empirical approach to treatment is preferred. Written informed consent from the child's parent or legal guardian and verbal assent of the child or adolescent (when doable, given cognitive delays) is necessary before any medication trial may begin. Target symptom approaches or diagnostic approaches to medication treatment in autism need to be explained. Objective behaviors and

quality of life measures need to be defined before the initiation of treatment and tracked during treatment. Reliable and valid rating scales or observational strategies should be used to measure target behaviors. Baseline observations and data should be collected to be used in comparison with observations and data collected during active treatment.

If possible, individual patient treatment—reversal single trial designs—are recommended. These involve on-off-on or off-on-off designs, in which medication is periodically removed and restarted, to test its safety and efficacy in relation to the management of target behaviors. Medications should be introduced as a single variable into the treatment plan, and not introduced with a variety of other treatments occurring at the same time. When medications are introduced as a single variable, their effects on target behaviors and their treatment emergent side effects are more clearly discernible.

The full dose range of a single drug should be explored (e.g., low, medium, high dose ranges) for an adequate length of time at each dose level (generally several weeks, but defined by knowledge of the specific medication) before clinically abandoning the medication and starting another or adding additional medications. The danger of ineffective polypharmacy, in which multiple drugs are each given at subtherapeutic doses for indeterminate lengths of time, is very real in the pharmacologic treatment of persons with autism. Finally, medications should be introduced into treatment for predetermined periods of time that have a clear beginning and ending. Medication treatment for youngsters with autism should not occur for indeterminate and lengthy periods of time without either periodically reassessing the clinical risks and benefits of the proposed treatment plan or objectively demonstrating evidence of a clear clinical response.

If medications are used for the empirical treatment of associated symptoms in children and adolescents with autism using this type of systematic approach, their clinical use becomes much more rational and there is an increased probability of assessing efficacy and minimizing adverse side effects compared with using medications in a nonmethodical manner. General principles of treatment are outlined in Table 7.1.

Medications: Review of Recent Studies

This section reviews recent studies involving a number of medications that are being used in the treatment of persons with autism and then presents a practical guide to intervention. We focus on reported studies conducted on individuals with either autism or PDD since 1990. Although it would be preferable to report only on methodologically controlled studies, such as randomized clinical trials, the paucity of this type of study with many of the medications used in

TABLE 7.1. Principles of Medication Management for Clinically Referred Children and Adolescents with Autism or Related Disorders

1. Use medication in autism based on the presence of a medication-responsive comorbid psychiatric diagnosis, a medication-responsive target symptom, or a behavioral-biologic rationale; and only after conducting a complete diagnostic and psychoeducational assessment.
2. Use medication for persons with autism only adjunctively within a coordinated multidisciplinary psychoeducational treatment plan.
3. Obtain written informed consent from the child's legal guardian and oral assent from children 7 years of age or older (if possible, given cognitive delays) for all medication trials.
4. When beginning empirical treatment for the child or adolescent with autism and emotional or behavioral problems, use the most benign interventions first.
5. When beginning medication treatment, have some quantifiable means of assessing the safety and efficacy of the drug:
   a. Define objective behaviors and quality-of-life measures to be tracked.
   b. Use empirical methods (validated, treatment-sensitive rating scales).
   c. Obtain off-drug baseline data and on-drug treatment data for comparison.
   d. Obtain data at regular intervals during drug treatment.
6. Institute medication trials systematically:
   a. Whenever possible, medication should be introduced as a single variable into treatment.
   b. Medication trials should be introduced for preplanned periods of time that have clear beginnings and endings, so that drug efficacy can be assessed.
   c. Explore the full dose range of a single medicine for an adequate length of time (generally several weeks) before switching to a different drug or adding medicines.
   d. Avoid polypharmacy with multiple drugs all given at subtherapeutic doses for inadequate periods of time.
   e. Follow drug serum levels where available.
   f. Assess side effects systematically.

persons with autism precludes this approach. Hence, we will present data from open-label trials as well. In the study of autism, single case reports, case studies, and open-label trials of pharmacologic interventions have resulted in great enthusiasm regarding the potential of a number of medications. However, well-controlled studies, and time, have frequently failed to confirm this initial enthusiasm. Therefore, a cautious, considered approach to the use of many of these interventions is indicated.

Note that studies have often been conducted separately in adults and in children. Studies of normal neurodevelopment suggest obvious and significant differences in brain structure and functioning across age groups. For example, there is evidence in the depression literature that children with depression may respond differently than their adult counterparts to antidepressant medications and that this difference may be at least partially related to underlying age-related neurophysiologic differences (Ryan, 1992; Birmaher et al., 1996). There is reason to believe that this may be true in autism as well. Autopsy studies of autistic individuals have demonstrated structural differences between the brains of children and adults with this disorder (Kemper and Bauman, 1998). Therefore, caution should be exercised in assuming that results of a completed study of medication intervention in adults with autism can be directly applied to children with the same disorder.

ATYPICAL ANTIPSYCHOTICS

Since 1990, eight studies have reported the use of atypical antipsychotic medication in the treatment of individuals with autism (Table 7.2). The agents studied have been risperidone (six studies; four open label; four studies involving children and adolescents; $N = 101$) (Findling et al., 1997; Horrigan and Barnhill, 1997; McDougle et al., 1998; Nicolson et al., 1998; Malone et al., 2002) and olanzapine (two studies; both open label; both included children and adolescents; $N = 20$) (Potenza et al., 1999; Malone et al., 2001). The atypical antipsychotic medications resperidone and olanzapine have received most of the attention due to (1) their presumptive capacity to stabilize behavior in a manner similar to the effect that has been observed with haloperidol (Campbell et al., 1996, 1999) and (2) a reduced risk for the development of long-term extrapyramidal side effects and tardive dyskinesia. Studies with these agents suggest overall improvement in the functioning of the subjects treated with these agents, but the saliency of these interventions appears to be their capacity to reduce hyperactive/impulsive, aggressive, and repetitive stereotypic behaviors. Because of their increased safety and tolerability compared to typical antipsychotics, atypical antipsychotics are the first choice for antipsychotic medication in persons with autism.

These medications are generally well tolerated in short-term treatment. Mild transient sedation and weight gain are the most commonly observed side effects reported in studies. However, there is significant concern over the velocity, extent, and consequences of the observed weight gain that may be associated with the use of atypical antipsychotics. Children and adolescents may be more vulnerable to antipsychotic-induced weight gain than are adults. Medical complications of weight gain include insulin-resistant type II diabetes and elevated

serum triglycerides and cholesterol, with attendant implications for long-term reduced cardiovascular functioning (Biswasl et al., 2001; Hagg et al., 2001; Henderson, 2002; Newcomer et al., 2002; Sernyak et al., 2002). Furthermore, concern regarding the long-term use of these agents is heightened by reports of tardive dyskinesia associated with risperidone use in children (Feeney and Klykylo, 1996; Kumar and Malone, 2000).

In summary, the atypical antipsychotics constitute a class of medications that appear to be effective in reducing interfering behaviors, such as aggression and stereotypies, in this population. Atypical agents are preferred over older, typical antipsychotics, because of their generally better safety profile. However, the numbers of subjects that have been studied is small and the duration of reported trials is typically short term. Thus, there is limited information about chronic exposure to these substances in this population, and there remains the potential for side effects that warrant careful monitoring.

ANTIDEPRESSANTS

The current interest in antidepressants in the treatment of autism rests on two scientific findings. The first finding concerns evidence that agents that effect serotonin transmission have the capacity to reduce obsessive-compulsive behaviors (Hollander, 1998). It has been hypothesized that these agents may be useful in reducing repetitive stereotypic behaviors commonly observed in autistic individuals. The second finding concerns evidence that there are anomalies in CNS serotonin transmission systems in autistic individuals (Cook and Leventhal, 1996; Chugani et al., 1997; Singh et al., 1997; Leboyer et al., 1999; Betancur et al., 2002). These findings have led to the investigation of agents known to alter serotonin availability, such as L-dopa and fenfluramine, and currently, the SSRI antidepressants.

With the exception of a small pilot study using imipramine in 1971 (Campbell et al., 1971), studies in autism have used antidepressants known to have a robust effect on serotonin transmission. Clomipramine is a tricyclic antidepressant with potent blockade of the presynaptic neuronal serotonin reuptake mechanism. Since 1990, two studies using this agent have been reported. In 1993, Gordan et al. reported on a double-blind crossover study in 24 autistic individuals (mean age: 10.4 ± 4.1 years) using clomipramine and desipramine (the latter a tricyclic antidepressant with minimal effect on serotonin transmission). The patients in the clomipramine group showed greater global improvement and reduction of repetitive behaviors than did those in the placebo or desipramine groups. Side effects in this study were minimal and included mild sleep disturbance and anticholinergic effects. However, clomipramine is an agent with mild

TABLE 7.2. Atypical Antipsychotic Trial in Autism and PDD

Study	*N*	*Age Group*	*Design*	*Drug/Dose Range*	*Duration (weeks)*	*Side Effects*	*Outcome*
Malone et al. (2002)	22	Child Adolescent	Open	Risperidone 0.5–4.0 mg/day	4 acute 24 long-term	Sedation, increased appetite, weight gain	Improvement noted on CPRS and CGI.
Malone et al. (2001)	12	Child Adolescent	Open	Olanzapine 2.5–20 mg/day Haloperidol 0.25–5 mg/day	7	Drowsiness, weight gain	Five of six olanzapine and three of six haloperidol subjects improved on CGI. Both groups improved on CPRS.
Potenza et al. (1999)	8	Child Adolescent Adult	Open	Olanzapine 2.5–20 mg/day	12	Increased appetite, weight gain, and sedation	Seven of eight completed trial; six of seven completers showed significant improvement on CGI.
McDougle et al. (1997)	18	Child	Open	Risperidone 1–4 mg/day	12	Transient sedation, weight gain	Twelve of 18 subjects showed significant improvement. Repetitive and aggressive behaviors reduced.
McDougle et al. (1998)	14[a]	Adult	DBPC	Risperidone 1–10 mg/day	12	Mild, transient sedation	Aggression, anxiety, depression, and irritability improved.

Nicolson et al. (1998)	10	Child	Open	Risperidone 0.05 ± 0.02 mg/kg/day (mean)	12	Transient sedation, weight gain	Eight of 10 rated improved on CGI, CARS, and PTQ.
Horrigan and Barnhill (1997)	11	Child Adolescent Adult	Open	Risperidone 0.5–1.5 mg/day	4	Weight gain, mild transient sedation, possible chemical hepatitis ($n = 1$)	Aggression, self-injury, explosivity, overactivity, and sleep improvements noted.
Findling et al. (1997)	6	Child	Open	Risperidone 0.03–0.06 mg/kg/day	8	Weight gain, sedation	Improvement noted on CGI and CPRS.

Note: CPRS = Children's Psychiatric Rating Scale; CGI = Clinical Global Impression; DBPC = double-blind placebo-controlled; CARS = Childhood Autism Rating Scale; PTQ = Conners Parent-Teacher Questionnaire.

[a] On active medication/completing trial.

proconvulsive properties. This study group was a population of autistic individuals with no clinical or electroencephalographic evidence of a seizure disorder. In spite of this fact, one patient was reported to have had a brief seizure, underscoring the need for careful clinical consideration before treating and careful monitoring of persons with autism with agents known to lower the seizure threshold. A subsequent open-label study of eight children failed to reveal any significant improvement compared to placebo, and side effects were a significant concern (Sanchez et al., 1996). Side effects in this study included severe constipation, drowsiness, behavioral worsening, insomnia, and urinary retention requiring catheterization in the youngest subject.

Beginning in the mid-1980s, the first substantially different class of antidepressants became available. These agents have typically been as effective in the treatment of depression as the original tricyclic antidepressants, but with better safety and tolerability profiles. This class of new agents, the SSRIs, has been studied in autism and includes fluoxetine, paroxetine, fluvoxamine, citalopram, and sertraline. SSRI antidepressants are known to have potent effects on serotonin transmission. Since 1990, seven studies have been reported in the literature (see Table 7.3). The medications used in these investigations have included fluoxetine (two studies; open label; principally children and adolescents; $N = 35$) (Cook et al., 1992; Alcami Pertejo et al., 2000), fluvoxamine (two studies; double blind; children and adults; $N = 48$) (McDougle et al., 1996; Fukuda et al., 2001), and sertraline (open study; open label; children; $N = 9$) (Steingard et al., 1997). These reports suggest that the SSRIs appear to reduce repetitive, stereotypic behaviors, obsessive-compulsive behaviors, anxiety, and aggression, with a secondary benefit of improving global functioning. Side effects in the studies of fluvoxamine and sertraline were minimal and included mild sedation and gastrointestinal (GI) discomfort, but the studies with fluoxetine also reported evidence of behavioral worsening in some of the individuals that resolved with either medication reduction or discontinuation.

STIMULANTS

Short attention span, impulsivity, and hyperactivity are symptoms that frequently accompany a diagnosis of autism. The mainstay of treatment for these symptoms in children with attention deficit hyperactivity disorder (ADHD) has been the stimulant medications methylphenidate, dextroamphetamine, and their new once-daily preparations. Although early studies of amphetamine treatment of autistic children suggested that these medications could result in the reduction of hyperactivity, many of the children in these investigations experienced clinically significant side effects, such as irritability and increased stereo-

typic movements. The use of stimulant medication was thought to be contraindicated. However, Birmaher et al. (1988) reported on the open-label use of methylphenidate to treat nine children with autism and symptoms consistent with ADHD: hyperactivity, inattention, and impulsivity. All the children demonstrated improvement in behavior at home, and only one child failed to show improvement at school. No significant side effects were reported. In 1995, Quintana and colleagues (1995) followed up this report with a double-blind crossover study using two doses of methylphenidate (10 mg or 20 mg bid) and placebo. The subjects experienced modest but significant improvements in behavior and hyperactivity with no reports of significant side effects, irritability, or increased stereotypies.

A recent review of pharmacologic treatments identified 10 reports regarding the effects of stimulants for hyperactive/impulsive behaviors in children and adolescents with autism or PDD (Aman and Langworthy, 2000). This review confirmed the presence of empirical evidence for the use of stimulants in the treatment of young individuals with autism or autism spectrum disorders and reported significant reductions in hyperactive/impulsive symptoms. In several of these studies, there is evidence that persons with developmental delay having an IQ > 55 or a mental age > 4.5 years responded best to stimulants (Aman et al., 1991a, 1991b). Based on the strength of these more recent studies, clinicians are increasingly comfortable prescribing stimulants to persons with autism who demonstrate moderate to severe ADHD symptoms and are mildly cognitively delayed.

OPIOID ANTAGONISTS

A dysfunction in opioid neurotransmission in autism is the theoretical rationale for the use of naltrexone, a pure opioid antagonist. Early interest in the opiate blockers was predicated by the notion that opiate blockers may either reduce the core symptoms of autism or reduce associated symptoms, such as self-injury and hyperactivity. Since 1990, nine studies have examined the safety and efficacy of naltrexone for psychiatric symptoms in children with autism (Panksepp and Lensing, 1991; Leboyer et al., 1992; Campbell et al., 1993; Kolmen et al., 1995, 1997; Willemsen-Swinkels et al., 1995a, 1995b, 1996; Feldman et al., 1999) (Table 7.4). Eight studies are controlled and one is an open study. The dose of naltrexone used in these investigations is generally 1.0 mg/kg. A number of these studies report a reduction in hyperactive behavior. However, these studies do not demonstrate a significant effect on learning, communication, or social behaviors in children with autism (Feldman et al., 1999). It has been suggested that future research should assess baseline anxiety and evaluate its capacity to moderate the response to naltrexone. Given naltrexone's positive outcome on impulsivity and

TABLE 7.3. Antidepressant Studies

Study	*N*	*Age Group*	*Design*	*Drug/Dose Range*	*Duration (weeks)*	*Side Effects*	*Outcome*
Steingard et al. (1997)	9	Child	Open	Sertraline 25–50 mg/day	2–52	Behavioral worsening, explosive and aggressive behavior	Eight of nine subjects clinically improved.
Alcami Pertejo et al. (2000)	12	Child	Open	Fluoxetine 15–20 mg/day	52	Impulsivity, restlessness, sleep difficulties, loss of appetite	11 children completed study. All experienced moderate or marked improvement.
Fukuda et al. (2001)	18	Child	DB cross	Fluvoxamine		No severe adverse effects	Improved language and eye contact. CGI improved in half of all subjects.
McDougle et al. (1996)	30	Adult	DBPC	Fluvoxamine 50–300 mg/day	12	Mild sedation, GI distress, nausea	Fluvoxamine is significantly better than placebo.
Sanchez et al. (1996)	7	Child	Open	Clomipramine 25–250 mg/day	5	Acute urinary retention, severe constipation, insomnia, behavioral toxicity, drowsiness	Clomipramine was not therapeutic and was associated with serious untoward effects in this sample.

Gordon et al. (1993)	24	Child	DB cross	Clomipramine 25–250 mg/day Desipramine 25–250 mg/day	10	Grand mal seizure in one subject, prolongation of the corrected QT interval, behavioral toxicity	Clomipramine is superior to both placebo and desipramine on ratings of autistic symptoms with no difference between desipramine and placebo.
Cook et al. (1992)	23	Adolescent	Open	Fluoxetine 20 mg every other day to 80 mg/day	1–61	Restlessness, hyperactivity, agitation, decreased appetite, insomnia	Significant improvement in CGI ratings of clinical severity in 15 of 23 subjects.

Note: CGI = Clinical Global Impression; DB = double-blind; QT = QT interval on EKG.

TABLE 7.4. Naltrexone Trials in Autism and PDD

Study	*N*	*Age Group*	*Design*	*Drug/Dose Range*	*Duration (weeks)*	*Side Effects*	*Outcome*
Feldman et al. (1999)	24	Child	DB cross	1.0 mg/kg/day	2	None reported	No improvement noted in communication.
Kolmen et al. (1997)	11	Child	DB cross	1.0 mg/kg/day	2	Drowsiness, decreased appetite	Modest improvement seen in behavior but no improvement in learning.
Willemsen-Swinkels et al. (1996)	23	Child	DB cross	0.74–1.18 mg/kg/day	4	None reported	No difference noted from placebo.
Willemsen-Swinkels et al. (1995b)	20	Child	DB cross	1.48–2.35 mg/kg	Single dose	None reported	No significant changes noted in social behavior; reduced irritability and target scores on behavior checklists.
Willemsen-Swinkels et al. (1995a)	32	Child	DB cross	50 mg/day or 150 mg/day	4	Acute, severe increase in SIB, nausea, tiredness	No clinical value observed.
Kolmen et al. (1995)	13	Child	DB cross	1.0 mg/kg/day	2	Drowsiness, aggression	Modest improvement of behavior and social communication noted.

Campbell et al. (1993)	41	Child	DBPC	0.5–1 mg/kg/day	6	Sedation, decreased appetite, aggression, hyperactivity, stereotypies	Naltrexone significantly reduced only hyperactivity and had no serious untoward effects.
Leboyer et al. (1992)	4	Child	DB cross	0.5–2 mg/kg/day	2	None reported	Children on lowest and highest doses displayed behavioral improvements.
Panksepp and Lensing (1991)	4	Child Adolescent	Open	0.4–0.8 mg/kg/day	3–52	Panic attacks at higher doses	Reduction in the positive symptoms of autism noted.

Note: DB = double-blind; DBPC = double-blind placebo-controlled; SIB = self-injurious behavior.

hyperactivity, naltrexone studies should use specific measures that directly address aspects of attention span and impulsivity in children with autism.

ALPHA-2 ADRENERGIC AGONISTS

Clonidine is a presynaptic alpha adrenergic agonist that down-regulates noradrenergic activity and outflow at the level of the midbrain locus ceruleus. Because persons with autism may have difficulty becoming overaroused, and because arousal is largely mediated by noradrenergic systems, there exists a rationale to treat symptoms of overarousal in persons with autism with clonidine. These symptoms may include attention deficits, impulsive and hyperactive behaviors, insomnia, excessive anxiety, aggressive behaviors, and/or self-injurious behaviors.

Two controlled studies have assessed the efficacy of clonidine for reducing hyperarousal and symptoms of ADHD in children with autism. Jaselskis et al. (1992) studied eight male children (average age 8.1 ± 2.8 years) with autistic disorder who had problems with inattention, impulsivity, and hyperactivity. Subjects in this study had not tolerated or responded to other pharmacologic treatments, including typical antipsychotics, stimulants, or tricyclic antidepressants. All children completed a placebo-controlled, double-blind crossover protocol for clonidine. Results showed that parents reported more improvement on clonidine than did teachers or clinicians. Parents reported modest benefits of clonidine on symptoms of irritability and hyperactivity; however, side effects of clonidine included drowsiness, sedation, and decreased activity. The authors concluded that clonidine was modestly effective in the short-term treatment of irritability and hyperactivity in some children with autistic disorder.

Frankhauser et al. (1992) reported on the effect of transdermal clonidine on symptoms of hyperarousal in nine autistic males aged 5 to 33 years. Subjects completed a placebo-controlled, double-blind crossover study involving 4 weeks of clonidine or placebo with a 2-week washout between treatment conditions. The average clonidine dose was 0.20 mg/day. Results showed that clonidine was effective in reducing hyperarousal in these individuals, and that these individuals experienced improved social behaviors when overarousal was moderated by clonidine.

No studies have been published using guanfacine, a more selective alpha adrenergic agonist, in persons with autism. Whether this medication will prove to be more effective than clonidine must await further research.

BETA-BLOCKERS

Beta-blockers are competitive antagonists of norepinephrine and epinephrine action at postsynaptic beta adrenergic receptor sites. These receptors are found in

both the central and peripheral nervous systems. In the periphery, β_1-receptors mediate cardiac (positive chronotropic and inotropic) effects, and β_2-receptors modulate bronchodilation and vasodilation. Beta-blockers differ in their selectivity for beta adrenergic receptors and in their lipid solubility (which determines peripheral or central nervous system site of action). For example, propranolol and nadolol are nonselective beta-blockers, acting on both β_1- and β_2-receptors. Atenolol and metroprolol are selective β_1-blockers. Propranolol is very lipid-soluble and readily enters the brain, exerting effects both centrally and peripherally. Atenolol and nadolol cross the blood-brain barrier in only small amounts and act primarily in the periphery. Other beta-blockers have intermediate degrees of lipid solubility (Connor, 1994). The rationale for the use of beta-blockers is based on the observation that persons with autism may become aggressive and hyperactive when overstimulated, and that arousal is largely mediated by epinephrine and norepinephrine mechanisms.

An open-label pilot study assessed the safety and efficacy of nadolol as an adjunctive pharmacologic treatment for aggression and hyperactivity in 12 children, adolescents, and young adults with PDD (Connor et al., 1997). Subjects were 9 to 24 years of age, developmentally delayed, and completed a 5-month open prospective protocol of nadolol with systematic baseline and outcome evaluations and weekly clinical assessments. The single-dose range used in this study was 30 to 225 mg/day (mean dose 109 mg/day). Results demonstrated that 10 subjects (83%) experienced a reduction in aggressive behavior. No significant effects were found for hyperactive behaviors.

SECRETIN

Secretin is a polypeptide neurotransmitter that is approved by the U.S. Food and Drug Administration (FDA) for single-dose intravenous use in assessing pancreatic function. In 1998, Horvath et al. (1998) reported on the use of secretin to stimulate pancreaticobiliary secretion in three children with autistic spectrum disorder. They reported dramatic improvement in behavior, eye contact, alertness, and expressive language within 5 weeks of the infusion. Since then 12 additional reports have been published on trials that used a single dose of secretin (Sandler et al., 1999; Chez et al., 2000; Dunn-Geier et al., 2000; Coniglio et al., 2001; Corbett et al., 2001; Owley et al., 2001; Carey et al., 2002; Kern et al., 2002; Molloy et al., 2002; Unis et al., 2002; Coplan et al., 2003; Levy et al., 2003). All of the studies were double-blind placebo-controlled studies (several groups employed a crossover design, and one group also conducted an open label trial). A total of 533 children were enrolled in these studies (323 received secretin). Only a single study has employed multiple doses (Roberts et al., 2001). In this study 64 children were recruited for a double-blind placebo-controlled trial in

which two doses were given 6 weeks apart. Thirty-two children received placebo injections and thirty-two children received secretin. All of the studies used structured evaluations of baseline behavior and response (e.g., Childhood Autism Rating Scale [CARS], Autism Behavior Checklist [ABC], Autism Diagnostic Observation Schedule [ADOS], and Autism Diagnostic Interview—Revised [ADI-R]). No appreciable differences were observed between the subjects treated with placebo and those who received a single dose of secretin in any of these studies. This finding was also reported in the multiple-dose trial. Although one study suggested that a subgroup of children with chronic active diarrhea had a preferential response to secretin, other studies have failed to replicate this suggestion.

Authors of these articles and their critics have pointed out numerous limitations in these studies. The summary by Dunn-Geier et al. (2000) suggests that, in spite of the limitations of any of these studies, the open label findings that initiated these investigations should have been replicated in the designs that were employed but were not. Although there may remain open questions regarding multiple injections and the effect on subpopulations of autistic individuals (e.g., less severely affected, presence of gastrointestinal problems), at this moment, secretin is an agent that should be employed only in controlled and monitored trials.

A Practical Guide to Psychopharmacology

THE CONTEXT OF TREATMENT

Treatment of autistic individuals often includes multiple services and providers. Although the treatment is centered on the clients and their families, a typical plan can include educational, medical, and behavioral interventions, physical therapy, occupational therapy, and speech and language interventions. All of these interventions are best conceived of as interlocking pieces of a puzzle that when working in concert may promote stability and improvement.

Given the absence of a cure, pharmacotherapy is relegated to a supporting role in the treatment of autistic individuals. The goal of pharmacotherapy is the reduction of behaviors that interfere with current functioning and/or the patient's capacity to participate in other treatment modalities and to enhance the patient's quality of life. Therefore, pharmacotherapy should take place in the context of other treatments that are offered, and interventions should be coordinated.

PSYCHOPHARMACOLOGIC EVALUATION

The evaluation of children with autism entails a complete review of information that includes (1) developmental history; (2) a medical history; (3) review of fam-

ily and social factors and supports; (4) school history; (5) physical and neurologic examinations; (6) speech/language/communication, audio and visual examinations; (7) psychological and cognitive assessments; and (8) occupational and physical therapy assessments.

Because pharmacologic evaluation occurs in the context of the development of a larger multimodal evaluation and treatment effort, the pharmacologic evaluation should focus on the question of appropriateness of a pharmacologic intervention and on defining the focus of that intervention. All data from concurrent or antecedent evaluations should be reviewed. If not already established, active liaison with other providers and school personnel should be established with the family's consent and involvement. Special attention should be given to a careful behavioral and developmental history, an exploration of possible contributing medical and nonmedical factors, and a list of potential target behaviors. The initial focus should be confirmation that all other alternative medical and nonmedical interventions have been considered or attempted and that any potential differential diagnosis has been considered and evaluated.

If medications are to be considered, it is critical that the family and all members of the treatment team are aware of the nature of the proposed intervention (i.e., medication, mechanism of action, dose range), the anticipated positive effects (target symptoms), the potential side effects, and the duration of the clinical trial. A fully informed consent process with the family and/or guardian is a critical component for establishing a viable treatment contract. Given that there is generally a paucity of controlled data for most proposed interventions and the limitations around pain recognition and communication that are part of autism, it should be made clear that any change in functioning should be reported immediately to the treating physician. Outcome measures should be clearly identified and shared with the family and treatment team, and a process for monitoring effects and side effects needs to be established. The frequency of follow-up will depend on the nature of the intervention, but in general, visits should be more frequent at the initiation of treatment or at moments of change in the treatment regimen.

AN APPROACH TO SPECIFIC SYMPTOMS

The most common requests for psychopharmacologic management in autistic individuals center on the management of (1) aggressive and self-injurious behaviors; (2) repetitive behaviors and obsessive-compulsive symptoms; (3) hyperactivity, inattention, and impulsivity; and (4) resistance to change and excessive fear. The following are brief recommendations regarding the management of each.

Aggressive and Self-Injurious Behaviors. Atypical antipsychotics, such as riperidone and olanzapine, appear to be the treatment of choice for this type of presentation. Alpha-2 adrenergic agonists, such as clonidine, may also be a useful adjunct. If the behavior occurs in the context of a strong family loading for bipolar disorder, mood stabilizers, such as lithium, may have a role in the treatment.

Repetitive Behaviors, Excessive Fear, or Anxiety/Resistance to Change. SSRI antidepressants, such as fluvoxamine or sertraline, appear to be effective in the treatment of this class of symptoms. Clomipramine can be used if these agents are not tolerated, but needs to be used with caution secondary to its side effect potential, such as adverse cardiac events, and should be avoided in individuals with known seizure risk.

Hyperactivity, Inattention, and Impulsivity. Treatment of these symptoms is similar to the treatment of ADHD. Stimulant medications are the first line agents for this population. Alpha-2 adrenergic agonists may be a useful alternative strategy, and atypical antipsychotics may be effective as well, but side effects preclude the use of these agents as a first line for this indication. Naltrexone may be useful for impulsivity and hyperactivity but further research is needed.

Summary

Evidence and clinical experience strongly suggest a role for carefully applied and monitored pharmacotherapy in the treatment of individuals with autism. When medications are used to treat individuals with autism, the resultant improvements are typically modest in nature when taken in the broader context of the disorder. However, these gains may be significant with regard to the impact they may have on the individual's access to important interventions, such as social and educational remediation.

Current practice typically combines aspects of the target-symptom approach and the "scientific method" approach in defining treatment goals and expectations. The focus of pharmacotherapy is on the reduction of symptoms that are impeding the individual's development, diminishing his or her quality of life, and detracting from his or her capacity to function in a variety of settings. Treatment decisions are an active collaboration between families, physicians, and often, a larger psychoeducational treatment team and should include a discussion of risks and benefits that are clearly elucidated before the initiation of an active intervention.

Well-controlled studies demonstrate that antipsychotics have been effective in reducing symptoms, but are associated with known toxicity. Preliminary evidence suggests that agents that alter serotonin neurotransmission may be of benefit in reducing repetitive behaviors and anxiety, and stimulants appear helpful with hyperactivity, impulsivity, and inattention. Such agents as α-2 adrenergic

agonists and neuropeptides have a role as second line agents and warrant further study. Secretin belongs in controlled trial settings until more data are acquired. More controlled trials in all classes are indicated.

After more than 40 years of work treating autism with medications, the field is still in its infancy. The advent of more sophisticated ways to study brain development and function and a growing emphasis on using this knowledge to inform new interventions bodes well for the future. Advances in the field will be based on a clearer understanding of the underlying neuropathophysiology that is responsible for the development of this disorder and the many symptoms that often accompany its presentation.

At the moment, however, the clinician is left with the skills that have always directed this type of intervention: command of the existing knowledge base, the capacity to listen well and observe, and the establishment of a stable treatment relationship with patients and their caregivers.

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8

Gastrointestinal Issues Encountered in Autism

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In 1943, Leo Kanner coined the term *autism.* The 11 children reported in this seminal paper were described as having unusual neurobehavioral presentations but were said to be otherwise healthy. Interestingly, eating disorders were identified in six of these children. Kanner believed these symptoms to be behavioral in nature rather than having a medical etiology. One child required a gastrostomy for nutritional support, and eventually the gastrointestinal (GI) issues were reported to have resolved spontaneously.

Over the next several decades, scattered references appeared in the literature suggesting a connection between nutritional factors and "neurobehavioral" symptoms. Prugh (1951), Daynes (1956), and Asperger (1961) suggested a link between behavioral disturbances and celiac disease (gluten sensitivity). Dohan (1966) hypothesized a relationship between gluten sensitivity and schizophrenia. In 1961, Crook et al. suggested that food allergies might play a role in a broad range of neurologic symptoms. Some of these early hypotheses have been discounted, partly because of limited data. However, there remains a great interest in the possible linkage between nutritional factors and neurobehavioral symptoms.

This chapter focuses on findings in the current GI/autism research while offering some speculation about the future course of exploring the GI/autism relationship. In the chapter, I discuss:

- GI problems in children with autism spectrum disorders (ASD)
- Dietary and nutritional factors in children with ASD
- Mucosal inflammatory conditions
- Functional bowel disorders
- Connections between behavioral issues and the GI tract
- Reflections on future paths of GI/autism research.

Overview of GI Problems in Children with ASD

GI function is profoundly complex, involving the gut (esophagus, stomach, the small and large intestines) and the central nervous system, all of which are affected by exterior factors: food intake, psychological influences, and the environment. In his book, *The Second Brain* (1998), Gershon discusses the concept of "neurogastroenterology." He describes the interaction between the big brain in the head and the nervous system of the GI tract (the second brain). We know little about the messages the gut sends to the brain and how that information is processed.

Questions constantly posed to the gastroenterologist treating children with ASD include: Is there a brain/gut connection in autism? Can GI symptoms contribute to autistic symptoms or behaviors? How often do GI problems present in children with autism? Are there GI problems specific to ASD?

In a retrospective study presented by Fombonne and Chakrabarti (2001), a medical record review of 261 autistic children found a history of GI symptoms in 18.8 percent. Taylor et al. (2002) reported that 17 percent of autistic children studied had associated bowel problems, including 9 percent with constipation, 1.4 percent with constipation and diarrhea, 4 percent with diarrhea, 1.5 percent with food allergy, and 0.04 percent reporting nonspecific colitis with ileal lymphoid hyperplasia. Fombonne and Taylor could be underestimating the prevalence of such conditions as food allergy and constipation in children with ASD. These two GI issues have a higher reported frequency in the general pediatric population studies (Yong and Beattie, 1998; Bernheisel-Broadbent, 2001).

Fombonne et al. (1997) reviewed associations of autism and various medical disorders. In an epidemiologic survey of 325,347 French children born between 1976 and 1985, autism was identified in 5.35/10,000 children. Compared to unaffected children, associated medical conditions were reviewed in the autistic children. The researchers found increased prevalence of epilepsy, fragile X syndrome, and tuberous sclerosis in children with autism. This study suggested that other previously associated conditions, such as congenital rubella, phenylketonuria, neurofibromatosis, and Down syndrome, did not show increased prevalence in the children with autism. GI conditions may be found to be more common in ASD, but so far, data to support this is lacking. Problem-focused, population-based studies are still needed.

Dietary and Nutritional Factors That May Be Seen in Children with ASD

Perhaps the greatest interest in the GI relationship to ASD focuses on the possibility that diet and nutrition play a direct or even indirect role in autism management. In this relationship, several food-related factors need to be considered:

- Allergy
- Food intolerance, as might be seen with inadequate digestion (e.g., lactose intolerance), which could be a cause of symptoms (abdominal pain, gas, and diarrhea)
- Deficiency of vitamins or building block substances affecting normal neurotransmission
- Pharmacologic effect of food and also the concept that poorly digested food by-products produce a neurologic effect.

FOOD ALLERGY AND CELIAC DISEASE

Food allergy and inflammation of the gut for other reasons could account for problems and symptoms in children with autism by several mechanisms. The GI symptoms of allergy could include pain, constipation, diarrhea, rash, and sleep disturbance. Because children with ASD have communication difficulties, they may not accurately define their complaints. Pain could be exhibited as outbursts, unexplained crying, or self-injury. Focusing on underlying medical issues may reduce the "behavior" by reducing triggers for that behavior.

Determining food allergy is difficult, and the various types of testing for allergy have pitfalls. Food elimination trials are fairly reliable at defining food intolerance, but cannot exact the diagnosis of food allergy, which, by definition, is an immune-mediated response (Sampson, 1999a, 1999b). Allergy can be evaluated by skin testing, IgE radioallergosorbent testing (RAST), and IgG RAST, among others. All tests risk identifying a clinically nonrelevant result and probably best serve as a guide to consider certain foods that could be an allergy culprit.

Evidence that concludes children with ASD have dietary allergy is often debated. Lucarelli et al. (1995) evaluated 36 patients with autism characterized by DSM-III-R criteria. These patients were evaluated by allergy skin testing and IgE levels, along with serum levels of IgG-, IgA-, and IgM-specific antibodies for cow milk and egg proteins. Her findings included positive skin testing in 36 percent of autistic children compared to a control group of unaffected children, which was positive in 5 percent. IgE level elevation was noted in 33 percent of the autistic children. In a separate study, Reichelt et al. (1990) found IgA-specific antibodies for gluten, gliadin, β-lactoglobulin, and casein in their evaluation of autistic patients.

Pursuing the question of the gut permeability and the potential for food sensitivity, D'Eufemia et al. (1996) evaluated 21 autistic children and 42 unaffected controls. The purpose of their study was to determine if increased passage of larger molecular-sized substances across the gut barrier was present in autistic children. They suggested that increased "leakage" of larger substances from the gut into the bloodstream might explain the potential for an increased sensitivity

to certain peptides or proteins. They found that 43 percent of the autistic children had increased permeability compared to none of the controls. These peptides in the bloodstream might then induce allergic sensitization or cause pharmacologic effects.

Gluten proteins, which are present in wheat, rye, barley, and malt, have been suggested to contribute to autism and behavioral problems. In addition to the possibility of specific allergy to these foods, celiac disease or gluten enteropathy may occur. Celiac disease is an immune sensitivity to gluten proteins, which requires lifelong elimination of these foods. Dohan (1966) suggested a link between the rising incidence of schizophrenia and the increased consumption of wheat-based cereals in northern Europe. Dohan et al. (1969) reported that relapsed schizophrenics showed more rapid clinical improvement of symptoms when placed on a milk- and cereal-free diet. Goodwin et al. (1971) evaluated 15 randomly selected autistic children, seven of whom had chronic diarrhea. These children were evaluated by electroencephalogram (EEG) with and without gluten exposure. The authors suggested that brain electrical activity was affected by gluten intake.

We must look with caution at early reports of a higher frequency of celiac or gluten sensitivity in autistic patients. The clinical observation that gluten foods affect the behavior of some children with autism should still be given consideration, but only recently have we been able to define celiac disease with more clarity. More recent research argues against a link between celiac disease and autism. Pavone et al. (1997) evaluated a limited number of autistic children (11) for markers of celiac disease and did not find any correlation. They also conducted evaluations on a large number of children (120) documented with identifiable celiac disease for any indication of behavioral abnormalities. They found that none of the children exhibited autistic-like behaviors, concluding there was no connection between celiac disease and autism. This study is far too small to generalize and a large population study is needed to better assess these findings.

Knivsberg et al. (1990) placed a selected group of autistic children in a residential school on a gluten-free diet and reported improvement. These children had been screened before the initiation of the diet and had been found to have evidence of a peptide, gliadomorphin, in the urine. The authors suggested that this finding identified children who may have a problem with gluten. The gluten-free diet prescribed in this study would have potentially been helpful for children with celiac disease, food allergy to gluten products, or maldigestion of these food products. The conclusions may support that at least a subgroup of autistic patients could benefit from dietary change. Sponheim (1991) offered a limited study evaluating autistic children placed on a gluten-free diet and observed no behavioral improvement. However, he did not qualify the partici-

pants by identifying a gluten sensitivity marker. Well-defined placebo-controlled trials to assess the potential benefits of a gluten-free diet are still needed.

There is often disagreement between parent and professional (Ho et al., 1994) over the management of children with autism and what constitutes therapeutic benefit. Many parents institute dietary restrictions and report anecdotally that various benefits have occurred. Validated tracking tools are lacking to assess progress from therapeutic or nutritional trials. These tools will be essential to answer the question: does diet make a difference?

DIETARY INTOLERANCE

Beyond allergy or immune-mediated food problems that may account for dietary contribution to behavioral problems, food intolerance or nonimmune reaction to foods could result from inadequate digestion of food (e.g., lactose intolerance). For 58 percent of the children evaluated, Horvath et al. (1999) described diminished lactase activity by intestinal biopsy of autistic subjects with diarrhea who underwent endoscopy. Our data support this finding: we reported (Kushak and Buie, 2002) 55 percent of affected children who underwent endoscopies for GI symptoms had diminished lactase activity by biopsy. In some children, other disaccharidase enzymes were diminished, including sucrase, maltase, and palatinase. The reported prevalence of lactose intolerance in the pediatric population falls below the frequency reported in the study by Horvath et al. (Kretchmer 1981). Possible reasons for enzyme deficiency might include intestinal injury or diminished genetic expression of these enzymes.

Horvath et al. (1999) measured pancreatic enzyme activity by performing secretin stimulation studies during endoscopy. They did not identify pancreatic enzyme deficiency in a limited number of children. More studies need to be performed to evaluate the possibility of pancreatic or intestinal malabsorption.

Alberti et al. (1999) suggested that poor breakdown of some food products could allow them to have a neurotransmitter effect, potentially accounting for altered behavior. They tested sulfation by dosing paracetamol (acetaminophen) in children with autism and measuring metabolites in the urine compared to an age-matched unaffected control group. The sulfated metabolite of acetaminophen was lower in autistic children, raising the question of whether the consumption of certain foods requiring sulfation processing exacerbates a metabolic dysfunction. Murch et al. (1993) discussed disruption of sulfation in intestinal inflammation (colitis). More recently, McFadden (1996) suggested that impaired sulfation has been found with increased frequency in individuals with several degenerative neurologic and immunologic conditions and might account for chemical and diet sensitivities in some children with autism.

NUTRITIONAL DEFICIENCY

Model research regarding vitamin supplementation and neurologic functioning has been conducted in the condition of tryptophan deficiency. Young (1991) looked at the effects of dietary components, including amino acids, carbohydrates, and folic acid supplementation, on behavior. He reported that tryptophan depletion decreased serotonin levels and affected mood. Other studies suggest that tryptophan depletion alters pain perception. Some types of pain may improve with supplementation of tryptophan; others, including postoperative pain, actually worsened when the patient was supplemented with tryptophan. McDougle et al. (1996) found that autistic symptoms worsened with tryptophan depletion.

Considering the model of tryptophan deficiency, the use of specific vitamin supplementation as a potential treatment for autistic symptoms has been debated. Researchers have reported that various supplements, including vitamin B6, vitamin B12, folic acid, calcium, magnesium, and zinc, may bring clinical improvement. One theoretical reason why folate, vitamin B6, and vitamin B12 may have value is that they are necessary for serotonin production. It is also possible that these agents may alter metabolic pathways or modulate gene expression. Folic acid deficiency has been identified in patients with depression (Young and Ghadiriam, 1989), schizophrenia (Godfrey et al., 1990), and affective disorders (Coppen et al., 1986). Vitamin B12 deficiency has been considered a potential contributing factor to neurologic dysfunction, perhaps because it aids protein synthesis and myelination (Herbert 1975). Lowe et al. (1981) could not identify deficiency of either folic acid or vitamin B12 in autistic patients.

Vitamin B6 (pyridoxine) supplementation has been reported to improve behavior in autism (Rimland, 1974). A subsequent double-blind, placebo-controlled trial supported the earlier report (Rimland et al., 1978). Discussing the metabolic approaches to the treatment of autism spectrum disorders, Page (2000) suggested that several well-designed studies, including Coleman (1989), Kleijnen and Knipschild (1991), and Lelord et al. (1988), supported the idea that pyridoxine improves some symptoms of autism. Speculation about mineral supplementation providing a benefit for autism is limited. Coleman (1989) supported calcium supplementation; Johnson (2001) discussed zinc supplementation. Plasma fatty acid levels, which are necessary structural components of neuronal cell membranes that modulate membrane function, were shown to be deficient in autistic children by Vancassel et al. (2001).

Many children with ASD receive vitamin and mineral supplements and other products every day on the basis of extremely limited data. This limited informa-

tion underscores the need for well-constructed clinical trials to evaluate these nutrients and better clarify the necessity and benefits of supplementation.

THE PHARMACOLOGIC EFFECT OF FOOD

In a pharmacologic review, Kromer (1988) delineated the manifold effects that opioid substances have on the GI tract. The effects include diminishing the peristaltic reflexes, modulation of acid secretion, increasing the release of histamine, and increasing luminal fluid resorption. Endogenous opioids may control developmental processes (Zagon and McLaughlin, 1978). Self-stimulatory and self-injurious behavior has been linked to β-endorphin levels (Barron and Sandman, 1983). Altered pain sensitivity has also been reported, and opioid antagonists—such as naloxone and naltrexone—have been used extensively in the treatment of children with autism in attempts to modulate opioid-associated symptoms (Panksepp, 1981). Sandyk and Gillman (1986) briefly focused on specific symptoms of autism that can be caused by abnormal endogenous opioid regulation, and Sahley and Panksepp (1987) delineated the idea that abnormal brain opioid activity could play a role in the genesis of many autistic symptoms.

Maldigestion of dietary peptides forms the basis of the opioid peptide theory of autism. Several investigators have proposed that maldigestion of dietary proteins, particularly casein and gluten-containing foods, produces small peptide molecules that may function as exogenous opioids. Peptides described as casomorphin (derived from milk) and gliadomorphin (derived from gluten foods) were identified in the urine of patients with schizophrenia and autism by Reichelt et al. (1991). These peptides were shown, in vitro, to bind to opioid receptors and therefore are speculated to affect the central nervous system by modulating opioid levels in the brain. Shattock et al. (1990) supported this theory. It is conceivable that the Opioid peptide theory could provide a possible explanation for reports of clinical improvement when some autistic children are placed on restrictive diets without proof of allergic sensitivity. One criticism of the theory, however, is the observation that these urinary peptides are present in asymptomatic children (Reichelt et al., 1998) and therefore may not have a physiologic effect.

Walker-Smith and Andrews (1972) described decreased expression of dipeptidyl-peptidase IV (DPP-IV) in a series of autistic subjects. This enzyme participates in the hydrolysis of peptide fragments of casein and gluten. DPP-IV, also known as CD-26, serves an immune function as a T-cell activator. There is speculation that DPP-IV is diminished in patients with autism. If DPP-IV activity is diminished, poor digestion of gluten and other food proteins could occur, allowing

"opioid peptides" to be present in excess. Presently, however, there is no study examining intestinal levels of DPP-IV in autism.

Mucosal Inflammatory Conditions Described in ASD

Information about gut inflammatory conditions in autistic children is profoundly limited, and reports are specific to ASD children with GI symptoms. In a limited study of 36 children with GI symptoms and ASD brought to endoscopy, Horvath et al. (1999) described esophagitis in 69.4 percent of ASD children undergoing endoscopy for GI symptoms. They also described gastritis in 42 percent and duodenitis in 67 percent of affected children.

Torrente et al. (2002) described duodenal biopsy findings in 25 children with "regressive" autism. The biopsy findings were compared to those of 11 celiac patients, five patients with mental retardation and cerebral palsy (MR-CP), and 18 control patients with normal histology. Twenty-three of 25 autistic children had normal histology or nonspecific increased cellularity. Immunohistochemical studies showed an overall marked increase in mucosal lymphocyte density in the autistic children compared to controls and MR-CP patients. The density of CD8 T-cells was also greater in the autistic patients than in controls and MR-CP patients. IgG deposition in the basolateral enterocyte membrane and subepithelial basement membrane was seen in 23 of 25 autistic children but was not seen in the control or MR-CP patients. The significance of these findings remains unclear, as this specialized testing is not usually done on all patients undergoing intestinal biopsy. Gastroenterologists may need to reconsider what we call normal biopsies and use these specialized tests more routinely.

Wakefield et al. (1998) reported the finding of inflammation in the lower GI tract in a group of children with PDD. Their study evaluated 12 children with "regressive developmental disorder." Eleven of the patients were reported to have colitis by histology, and all had the endoscopic finding of prominent lymphoid nodules in the ileum (lymphoid nodular hyperplasia [LNH]), colon, or both. The authors suggested that measles virus from vaccine potentially caused these intestinal changes. More recently, in a larger sample of subjects Wakefield et al. (2000) evaluated a group of 60 children with "regressive" autism and a control group of unaffected children undergoing colonoscopy. In the affected group, 93 percent had ileal LNH, whereas 14.3 percent of the unaffected children showed these changes. Histologic colitis was seen in 88 percent of the affected children but only 4.5 percent of the unaffected subjects.

It should be noted, however, that LNH has been found in conditions other than autism (Sabra et al., 1998). For example, allergy has been associated with LNH. Kokkonen and Karttunen (2002) reported that LNH was seen in the colon

in 46 of 140 children undergoing colonoscopy for persistent and severe GI symptoms. These children were not noted to have autism or other developmental issues. Ileal LNH was also seen in 53 of 74 children tested, suggesting that LNH is common in children and not specific to the ASD population. And it has been suggested that LNH may be an expression of immune response (Kokkonen and Karttunen, 2002).

Furlano et al. (2001) reported that, although the histologic findings of colitis described in autistic children were less severe than classical inflammatory bowel disease (IBD), immunohistochemical testing showed increased basement membrane thickness and mucosal gamma delta cell density compared to IBD. The significance of these findings is unclear. Wakefield et al. (2002) suggested that the presence of colitis or LNH may change intestinal permeability and therefore allow opioid peptides to alter the neurologic status in these children. They compared the symptoms seen in autism to those associated with encephalopathy. In addition, they analyzed the ileal tissue for the presence of measles virus RNA. Ongoing research is continuing to evaluate the reports of colitis in ASD patients.

A large number of papers have been published refuting the idea that measles or measles-mumps-rubella (MMR) vaccine administration is associated with the development of autism. Taylor et al. (2002) saw no change in the frequency of regression before and after the institution of MMR vaccination in England. They did note a possible association between nonspecific bowel problems and regression, but did not find that this observation related to MMR vaccination. Fombonne and Chakrabarti (2001) evaluated patient groups before and after the institution of MMR vaccination. They found no difference in the age of the first parental concern for autism in children who are exposed to MMR than children who are not exposed and noted that the rate of developmental regression did not differ before and after MMR vaccine. The interval from MMR vaccine to parental recognition of autistic symptoms was comparable in autistic children with or without regression. Halsey and Hyman (2001:13), reviewing data for the American Academy of Pediatrics, concluded that "although the possible association with MMR vaccine has received much public and political attention . . . the available evidence does not support the hypothesis that MMR vaccine causes autism or associated disorders or IBD. A potential subgroup of children affected could not be excluded by this review."

Other considerations of impaired bowel health include an unhealthy bowel flora. Sandler et al. (2000) suggested that disruption of indigenous gut flora might promote colonization with bacteria that produce neurotoxins. They treated autistic children with oral vancomycin, and eight of 10 subjects showed transient behavioral improvements. Brudnak (2002) and Linday (2001) described the potential use of probiotic (nonpathogenic bacteria and yeast) agents, such as

Lactobacillus species and *Saccharomyces boulardii.* The value of these organisms may be to normalize intestinal flora, provide digestive enzymes that aid nutrient absorption, minimize allergen exposure, and stimulate local immune responses in the intestine.

Evaluating children with ASD and GI symptoms, Horvath et al. (1998) performed endoscopy and pancreatic function testing by administering secretin. Secretin is a neurotransmitter produced in the duodenum that stimulates the washout of pancreatic enzymes into the intestine. Following the procedure, he reported improvements in social behaviors, including better eye contact, increased social awareness, and improved expressive language in three children. A flurry of studies followed, in efforts to assess whether secretin had a therapeutic benefit for the greater community of autistic children. Sandler et al. (1999), Lightdale et al. (2001), Roberts et al. (2001), and others reported no sustained GI or neurologic benefit of secretin over placebo. In addition, more recent phase III FDA therapeutic trials did not find a benefit of secretin over placebo (Repligen, unpublished data).

Functional Bowel Disorders

Functional GI issues include gastroesophageal reflux, recurrent abdominal pain, and irritable bowel syndrome (IBS). Population-based survey studies remain sorely lacking to fully determine how often children with ASD have GI function problems. We cannot speculate on how frequently this will be seen in ASD, but should not assume that these conditions happen less frequently in the nonverbal child than in the verbal child. Such conditions as recurrent abdominal pain, abdominal migraine, and IBS have no defining test to clarify the diagnosis. Symptom history is the best current method to identify these disorders and therefore may be difficult to ascertain in this population, as many children with ASD exhibit significant sensory derangement. Symptoms seen in sensory integration disorder, such as altered pain perception, are a primary component of IBS as well and may be a reason to consider this diagnosis in children with GI issues and ASD.

Behavioral Issues with Specific Impact on the GI Tract

Burd et al. (1995) described children with ASD who exhibited food selectivity or textural selectivity. Feeding difficulties are common (>30%) in children with developmental delays (Gouge and Ekvall, 1975), but reports of mealtime or feeding problems in unaffected children are common as well (Bentovim, 1970).

Ahearn et al. (2001) evaluated 30 ASD children with reported atypical eating behaviors. They found type or texture selectivity in 17 of 30 children. Palmer et al. (1975) used applied behavioral analysis in a child with food selectivity. Using a behavioral treatment plan, acceptance of a normal variety was achieved and maintained. This study makes the point that medical reasons should be considered when there is prolonged subsistence on pureed foods, delay or difficulty in sucking, swallowing, or chewing, and delay in self-feeding.

Toilet training is often delayed in ASD, as in other developmental disorders (Dalrymple and Ruble, 1992). Of the surveyed patients with a mean age of 19.5 years, 22 percent did not have full success with toileting. Underlying medical issues, such as constipation, may present a barrier to successful toilet training.

Speculations and Conclusions

Berney (2000:20) states, "In the absence of a cure, the implementation of ideas will continue to outstrip factual evidence." Some pursuits that remain worthy include:

- Continued efforts to obtain well-designed population-based information in ASD that may help define phenotypic subgroups for which medical interventions can be selected
- Development of comprehensive tools, such as a disease activity index (as in IBD), or quality-of-life measures to track changes in behavior and development to determine the potential benefits of diet, nutrient, or medical interventions
- Ongoing work to determine biomarkers that can identify affected children who may have GI disturbance, and to develop noninvasive tools to screen for children who may benefit from more formal GI testing
- Development of an understanding of sensory and pain mechanisms and how to best characterize them in children with communication disorders
- Search for neuroimmunoregulatory factors in ASD and how these relate to GI inflammatory and allergy conditions in some children
- Education of health professionals to look for underlying medical issues in children with ASD.

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9

Structural Brain Anatomy in Autism: What Is the Evidence?

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It has been more than 60 years since Kanner (1943) first described autism in a series of 11 children who presented with a cluster of symptoms that included poor social relatedness, obsessive and stereotypic behaviors, and disordered language. In DSM-III (American Psychiatric Association, 1980), the term "pervasive developmental disorder" (PDD) was introduced to describe more mildly affected individuals with autistic-like features associated with disordered or distorted behavioral and cognitive development. Although initially believed to be distinctly different disorders, there is now a growing consensus that PDD and autism are part of a continuum (Allen et al., 1988), and the term "autism spectrum disorder" (ASD) has come into common usage.

Given the complexity of clinical features described in ASD, it is not surprising that multiple sites of brain abnormality were implicated by early investigators as being involved in this disorder. Suspected sources of abnormality included the vestibular system (Ornitz and Ritvo, 1968), the medial temporal lobe (Boucher and Warrington, 1976; Damasio and Maurer, 1978; Delong, 1978; Maurer and Damasio, 1982), the basal ganglia (Vilenski et al., 1981), the thalamus (Coleman, 1979), and the limbic system (Darby, 1976). More recent studies, however, have focused primarily on abnormalities in brain size and neuropathologic changes in the limbic and cerebellar circuits.

Gross Anatomy and Brain Size

The appearance of the autistic brain has been found to be unremarkable in most cases, both by neuroimaging techniques and by pathologic observation. There has been, however, a growing appreciation that individuals with autism may have unusually large head circumferences for age and sex, despite having had a normal head circumference at birth (Mason-Brothers et al., 1987; Courchesne et al., 2001). Magnetic resonance imaging (MRI) studies have shown variable find-

ings in regard to whether this "enlargement" is regionally specific or a more generalized process (Sparks et al., 2002). Piven et al. (1997), for example, suggested that there may be relative sparing of the frontal lobes. Courchesne et al. (2001) described the increase in brain volume as appearing between 2 and 4 years of age. In comparison, older children, aged 5–16 years, have brain volumes that were smaller than in controls. These authors noted that this early volumetric increase was primarily related to enlargement of both cerebral and cerebellar white matter, although some increase was also seen in cerebral gray matter. In a similar study, Aylward et al. (2002) measured brain volume in a series of high-functioning autistic subjects, 8–46 years of age. In this study, brain volume was noted to be significantly increased in autistic children, aged 8–12 years, but not in subjects older than 12 years of age when compared with controls. More recently, increased white matter volume in the brains of autistic subjects has been confirmed with MRI by Herbert et al. (2003), but with some suggestion of decreased gray matter volume. Given that the youngest child in three of these studies was 2 years old, it is not known whether the observed volumetric increase begins earlier in life, perhaps at a time when the clinical features of the disorder were beginning to become apparent. However, the combined results of these reports suggest that, relative to controls, brain development in autism is associated with an abnormally accelerated growth early in life, resulting in brain enlargement during childhood, followed by an apparent later deceleration in the rate of brain growth.

These data coincide with our own observations. We obtained fresh brain weights for 12 autistic children, aged 5–13 years (Table 9.1), and eight adults, ages 18–54 years of age (Table 9.2), and compared them with data from control subjects of the same age and sex. The majority of the childhood brains were significantly heavier than expected for age and sex, by 100–200 grams. In contrast, with the exception of one case, the adult brains were not significantly different from those of controls who were matched for age and sex (Bauman and Kemper, 1997).

There have been a number of attempts to more precisely localize abnormalities in the autistic brain with neuroimaging techniques. Inconsistent abnormalities of the cerebellar vermis have been reported by a number of investigators (Courchesne et al., 1988, 1994; Holttum et al., 1992; Kleiman et al., 1992; Piven et al., 1992) and in the brainstem (Gaffney et al., 1988; Hsu et al., 1991; Hashimoto et al., 1995; Piven et al., 1997). An early pneumoencephalographic study (Hauser et al., 1975) implicated medial temporal lobe structures. Although many subsequent computerized tomographic (CT) and MRI studies did not support abnormalities in this region (Piven et al., 1998), more recent investigations have shown in vivo evidence of a reduction in the size of the amygdala (Aylward et al., 1999) and hippocampus (Saitoh et al., 2001). It is likely that with improved technology, the delineation of specific brain regions in autism will continue to be an active area of research.

TABLE 9.1. Comparison of Brain Weights in Autistic Children from 5 to 13 Years Old

Sex	*Age (years)*	*Brain Weight (grams)*	*Expected Brain Weight (grams)*[a]	*Difference*
M	5	1,250	1,300	–50
M	5	1,480	1,300	+180
F	6	1,300	1,210	+90
M	7	1,504	1,330	+174
F	7	1,306	1,210	+96
M	8	1,542	1,370	+172
M	9	1,340	1,370	–30
M	9	1,670	1,370	+300
F	10	1,375	1,260	+115
M	11	1,332	1,440	–108
M	12	1,380	1,440	–60
F	13	1,350	1,280	+70

[a] Data from Dekaban and Sadowsky (1978).

TABLE 9.2. Comparison of Brain Weights in Autistic Individuals from 18 to 54 Years Old

Sex	*Age (years)*	*Brain Weight (grams)*	*Expected Brain Weight (grams)*[a]	*Difference*
F	18	1,382	1,340	+42
M	19	1,448	1,450	–2
M	20	1,290	1,450	–160
F	21	1,000	1,310	–310
M	22	1,280	1,440	–160
M	27	1,690	1,440	+250
M	28	1,210	1,440	–230
M	54	1,300	1,410	–110

[a] Data from Dekaban and Sadowsky (1978).

Microscopic Investigations

Without evidence of consistent gross brain abnormalities, there was little to guide the early microscopic analysis of the autistic brain. One of the first efforts was reported by Aarkrog (1968), who described a "slight thickening of the arterioles, a slight increase in connective tissue in the leptomeninges and some cell

increase" in a frontal lobe biopsy. Darby (1976) surveyed autopsy material from 33 cases of childhood psychosis and, as a result, suggested a possible relationship between limbic system lesions and the affective component of autism, but no specific pathologic abnormalities were described. Williams et al. (1980) studied brain tissue obtained from four subjects with autistic features but found no consistent microscopic abnormalities. Systematic counting of glial and neuronal cell numbers in multiple cortical regions of a single autistic brain and two control cases yielded similar results (Coleman et al., 1985).

Since that time, a variety of microscopic observations have been reported. Ritvo et al. (1986) noted decreased numbers of Purkinje cells in the cerebellar hemispheres and vermis in four autistic subjects when compared with controls. Slight thickening of the meninges, mild ventricular dilation, thinning of the corpus callosum, scattered perivascular lymphocyte infiltrates, and a few microglial nodules in the lower brainstem were reported in the brain of a 16-year-old autistic boy with mental retardation by Guerin et al. (1996). Additional brainstem abnormalities have been described in one autistic subject with Moebius syndrome, which included marked reduction in the number of neurons in the facial nucleus and superior olive and a shortening of the brainstem between the inferior olive and the trapezoid body (Rodier et al., 1996). The authors suggested that these findings indicate that these abnormalities might have their onset at around the time of neural tube closure.

Bailey et al. (1998) published observations in six mentally handicapped autistic subjects. Findings included abnormalities of neuronal density and laminar pattern in the frontal cortex of four of the six subjects. Decreased numbers of Purkinje cells and some patchy gliosis were found in the cerebellum, as well as ectopic neurons in the cerebral white matter and abnormalities of the inferior olive, ectopic olivary nuclei, and enlarged arcuate nuclei in the brainstem. Similar observations were reported by Weidenheim et al. (2001), who studied samples from five autistic brains and two cases with Asperger syndrome. In these cases, abnormalities of cortical lamination, heterotopic white matter neurons, and astrocytic proliferation were found in five of the seven cases; gliosis was noted in the olivary complex in two of the brains.

Using a technique of computerized analysis of brain sections, Casanova et al. (2002) studied cell microcolumn morphology in areas 9 (prefrontal cortex) and 21 and 22 (temporal lobe) of nine autistic brains in comparison with identically processed controls and observed small, more frequent, and less compact minicolumns in the autistic brains. These investigators suggested that changes in the width of minicolumns could affect the organization and processing of information.

In 1985, we reported the results of a systematic study of the entire brain of a 29-year-old well-documented man with autism, in comparison with an identi-

cally processed age- and sex-matched control, using the technique of whole-brain serial section (Bauman and Kemper, 1985). Since that time, we similarly and systematically studied eight additional clinically well-documented autistic cases and have begun to analyze an equally well-documented individual with Asperger syndrome.

Eight of the 10 brains were cut midsagittally with one-half of the brain available for study, and in two cases, the entire brain was studied. The autistic subjects studied fell into two age groups, including six children (five boys, one girl), aged 7–12 years, and three men, aged 22–29 years. The single male Asperger patient was 21 years of age. The autistic, Asperger, and control brains were identically processed and serially sectioned in the coronal plane. The initial studies involved the systematic analysis of the autistic and control material using a comparison microscope, in which corresponding brain areas were viewed side by side in the same field of view at the same magnification. In areas of these brains that appeared to be abnormal, neuronal cell size and the numbers of neurons per unit volume (cell packing density) were measured. However, our more recent quantitative studies of cell size and cell packing density have used computerized stereologic techniques (Gundersen et al., 1988; West and Gundersen, 1990; West, 1993; Coggeshall and Lekan, 1996).

As with previously reported autopsy cases, neither the autistic nor the single Asperger brain showed any gross abnormalities. The tinctorial density of myelin staining was comparable to controls in all brains. In contrast to observations by other investigators, multiple analyses of cerebral cortical laminar patterns throughout the serial sections revealed only three minor atypical findings. One was an indistinct laminar pattern in a small discrete area of the anterior cingulate gyrus and the other a minor malformation in the orbitofrontal cortex of one hemisphere (Kemper and Bauman, 1998). A third case showed scattered clusters of what appeared to be mineralized heterotopic neurons in the cerebellar molecular layer (Bauman and Kemper, 1987).

Systematic surveys of the forebrain showed no abnormalities of the striatum, pallidum, hypothalamus, basal forebrain, or bed nucleus of the stria terminalis. Preliminary observations in the ventral lateral thalamic nucleus in two autistic brains have shown small cell size and increased cell packing density in comparison to controls (Schultz et al., 1999). This nucleus has been implicated in circuitry involving abnormal serotonin metabolism in autism (Chugani et al., 1997).

With the exception of the abovementioned observations, forebrain abnormalities in all of the autistic brains appeared primarily to involve the limbic system. Small neuronal cell size and increased cell packing density were evident throughout the hippocampal complex, subiculum, entorhinal cortex, amygdala, medial septal nucleus, and mammillary body when compared with controls. Preliminary observations in the hippocampus of a single patient with Asperger

syndrome indicate similar findings to those observed in the autistic brains, but are less extensive (Madan et al., 2001). Analysis of individual hippocampal neurons in autism has been conducted using the Golgi technique. Reduced complexity and extent of dendritic arbors characterized these cells, most significantly in the CA4 and CA1 regions (Raymond et al., 1996). The findings of small cell size and increased cell packing density also characterized the nuclei of the amygdala, with the most pronounced abnormalities involving the medially placed medial, central, and cortical nuclei. The lateral nucleus of the amygdala was comparable to that of controls in all brains, with the exception of the 12-year-old child with normal IQ and behavioral outbursts (Bauman and Kemper, 1990) and in the brain of a 21-year-old man with Asperger syndrome (Bauman and Kemper, unpublished data), in which the lateral nucleus was diffusely involved. All forebrain areas found to be consistently abnormal in these autistic brains are known to be connected by closely interrelated circuits and comprise a major part of the limbic system of the forebrain.

In the septal region, however, a different pattern of abnormality was observed in the vertical limb of the nucleus of the diagonal band of Broca (NDB). In all of the childhood cases (less than 12 years of age), the neurons of the NDB were noted to be unusually large and abundant. In contrast, the neurons of this same nucleus were small and markedly reduced in number in all subjects older than 18 years of age (Bauman and Kemper, 1995).

The remaining consistent abnormalities in the autistic brain primarily involve the cerebellum and related cerebellar nuclei and inferior olive. All autistic brains studied, regardless of age, sex, and cognitive ability, as well as the Asperger brain, have shown a significant decrease in the number of Purkinje cells throughout the cerebellar hemispheres, with the most marked decrease occurring in the posterolateral neocerebellar cortex and the adjacent archicerebellar cortex (Arin et al., 1991). Only two of the autistic cases also showed pallor of the granule cells that occurred in areas of marked decrease in the number of Purkinje cells. Contrary to observations of vermal pathology noted in some imaging studies, no abnormalities in the density of Purkinje cells was found in the vermis (Arin et al., 1991; Bauman and Kemper, 1996).

In addition to the reduction of the number of Purkinje cells, abnormalities were also observed in the fastigeal, emboliform, and globose nuclei in the roof of the cerebellum, which appear to differ with age. Similar to the findings in the NDB in the septum, small pale neurons, which were decreased in number, characterized these nuclei in all subjects older than 18 years. In all of the autistic subjects younger than 12 years, the neurons of these nuclei, as well as those of the dentate nucleus, were noted to be enlarged and present in adequate numbers (Bauman and Kemper, 1994).

In the brainstem, abnormalities involved the neurons of the inferior olivary nuclei, areas known to be related to the abnormal cerebellar cortex (Holmes and

Stewart, 1908). Similar to the findings in the cerebellar roof nuclei and NDB of the septum, the neurons of the inferior olive in all of the autistic brains less than 12 years of age were significantly enlarged and plentiful when compared with controls. These same cells in autistic subjects older than 21 years were present in adequate numbers but were small and pale (Anderson et al., 1993). Furthermore, the neurons that showed abnormalities in cell size also failed to show the expected retrograde cell loss that invariably occurs following perinatal and postnatal Purkinje cell loss in human pathology (Norman, 1940; Greenfield, 1954). In addition, in all of the autistic brains, regardless of age, the neurons of the inferior olive tended to cluster at the periphery of the nuclear convolutions, a pattern observed in some syndromes of prenatal origin associated with mental retardation (Sumi, 1980; DeBassio et al., 1985).

Discussion

Neuroanatomic observations in the brains of our nine autistic subjects and preliminary findings in one brain of an Asperger patient have shown abnormalities, which primarily involve the limbic system and cerebellar circuitry and are the most consistent findings in our material. Although the observed abnormalities are consistent within our own sample, they at times differ in location and in pathologic description from observations reported by other investigators (as noted above). These differences may be due to different research techniques used for study, the amount and quality of brain tissue available for study, the degree of clinical severity of the subjects, and variations in phenotypic expression.

In spite of these concerns, our studies and the findings reported by other investigators indicate that at least five neuropathologies may be present in the autistic brain. These include: (1) increased brain weight and white matter volume during childhood; (2) reduced neuronal size and increased cell packing density in the forebrain limbic system; (3) reduced numbers of Purkinje cells in the cerebellum; (4) age-related changes in cell size and number in the nucleus of the diagonal band of Broca, deep cerebellar nuclei, and inferior olive; and (5) malformations of the cerebral cortex and brainstem.

Age-related changes in brain weight and volume have only recently been appreciated. Several studies have documented that the majority of children later diagnosed with autism have a normal head circumference at birth (Mason-Brothers et al., 1987; Courchesne et al., 2001). Sequential measurements of head circumference during the first several years of life suggest that the abnormal increase in head size becomes evident by 18 months of age (Bauman, unpublished data). Although there is growing evidence of increased white matter volume with neuroimaging techniques, both increased and decreased gray matter volume has also been reported.

A number of hypotheses have been suggested to explain the source of this early brain enlargement. It has been suggested, for example, that the observed macrocephaly may be secondary to the presence of increased numbers of cortical cell neurons, resulting from either augmented cell replication or impaired programmed cell death (Bailey et al., 1998). Although increased cell packing density has been reported in selected portions of the autistic limbic system (Bauman and Kemper, 1994), there has been no evidence that a similar pattern is present in the cortex (Coleman et al., 1985). Failure of the paring back of the neuronal neuropil has also been hypothesized. However, Golgi studies of individual hippocampal neurons instead show stunting of the dendritic arbors, a pattern consistent with developmental curtailment (Raymond et al., 1996) and indicating the probable presence of reduced amounts of neuropil, not more. The more likely explanation for the increase in brain size is the presence of improperly formed or chemically abnormal myelin, or an atypical up-regulation in the normal early production of myelin during the first years of life. A disturbance of myelin could lead to dysfunctional processing of information throughout the brain. Although no abnormalities of myelin have been observed with neuroimaging or pathologic analysis, preliminary neurochemical studies suggest the existence of quantitative differences in myelin phospholipids and glycolipids (Koul, 2001) and indicate the need for further research into the structure and biochemistry of myelin in autism.

The hippocampal complex, entorhinal cortex, amygdala, anterior cingulate, mammillary body, and medal septal nucleus are major parts of the limbic system. In the autistic brain, all of these regions show small and densely packed neurons without evidence of structural dysmorphology or abnormal distribution. The presence of small nerve cell bodies and reduced complexity of the neuropil are consistent with an earlier stage of maturation and suggest a curtailment of normal neuronal development. All of the limbic system areas noted to be abnormal in autism are part of the brain circuitry known to be important for memory and learning, emotion, and behavior (Papez, 1937), disturbances of which comprise some of the core clinical features of the disorder.

The observation of diffuse amygdalar involvement in a high-functioning autistic boy and a young man with Asperger syndrome, both cognitively normal but with deficits in pragmatic language and behavior, is intriguing. In contrast, in these same two brains, the findings of small cell size and increased cell packing density throughout the hippocampal complex was very mild relative to that noted in more functionally impaired subjects. These morphologic observations raise the possibility that variation in symptoms may be reflected in the extent to which parts of the limbic system are affected and may provide support for the concept of "autism" as a clinical and neurobiologic continuum.

The finding of reduced numbers of cerebellar Purkinje cells has been the most consistently observed microscopic feature in the autistic brain, regardless of age,

sex, and cognitive ability, and has been reported by numerous investigators. The decrease in the number of these cerebellar cortical cells appears to represent a congenital event, based on two observations. First, the reduced numbers of cells have occurred without evidence of obvious gliosis, which usually accompanies similar lesions occurring just before or after birth. Second, there has been a failure of the expected retrograde loss of the inferior olivary neurons, which typically occurs following perinatal or postnatal Purkinje cell damage in animals (Brodal, 1940) and in neonatal and adult humans (Holmes and Stewart, 1908; Norman, 1940; Greenfield, 1954). The occurrence of the retrograde olivary cell loss is believed to be secondary to the close relationship between the olivary climbing fiber axon and the Purkinje cell dendrites (Eccles et al., 1967). Inferior olivary climbing fibers have been shown to initially synapse with Purkinje cells in a transitory zone, called the *lamina dissecans,* located beneath the Purkinje cells (Rakic, 1971). Because this zone disappears in the human fetus between 29 and 30 weeks gestation (Rakic and Sidman, 1970), it is likely that whatever caused the reduced numbers of Purkinje cells occurred at or before that time.

The relationship of the cerebellar findings to those in the forebrain and to the clinical features of autism is unclear. Research in animals has shown the presence of a direct pathway between the fastigial nucleus of the cerebellum and the amygdala and septal nuclei, and a reciprocal connection between this nucleus and the hippocampus, suggesting that the cerebellum may play a role in the regulation of higher cortical thought and emotion (Heath and Harper, 1974; Heath et al., 1978). Additional studies have suggested that the cerebellum is a modulator of many central nervous system functions. It has been implicated in the control of shifting attention (Courchesne et al., 1992), as well as anticipatory planning (Leiner et al., 1987), mental imagery, and some aspects of language processing (Peterson et al., 1989), all of which have been reported to be impaired in autistic individuals. In addition, the cerebellum is believed to be involved in the regulation of the speed, consistency, and appropriateness of mental and cognitive processing (Schmahmann, 1991); it may have an impact on every level of behavior, including emotion and motivation, and may be involved in the control and integration of motor and sensory information (Schmahmann, 2001a, 2001b), functions that are also frequently disordered in autism.

A fourth pathology that appears to characterize the autistic brain is the presence of unusual age-related changes in cell size and number of neurons in the deep cerebellar nuclei, the inferior olive, and the NDB in the septum. In all of the children's brains (aged 5–13 years), these neurons were consistently enlarged and were present in adequate numbers. In contrast, in the older brains, the cells of the fastigeal, globose, and emboliform nuclei of the cerebellum and the NDB were observed to be small, pale, and markedly reduced in number. The neurons of the dentate and olivary nuclei, although small and pale, are not reduced in

number. Neuronal swelling followed by atrophy and cell loss is known to follow transection of an axon and has also been observed as a transneuronal event in the inferior olive following lesions of the central tegmental tract or dentate nucleus (Gautier and Blackwood, 1961). The presence of these microscopic characteristics, combined with changes in brain weight and volume with age, suggests that the pathologic changes in autism may include an ongoing postnatal process that affects brain connectivity. Clinically, there is as yet no evidence of a corresponding degenerative process. Elsewhere we have speculated that these postnatal age-related changes may represent the autistic brain's attempt to compensate for atypical circuitry (Kemper and Bauman, 1993).

Malformations of the neocortex have been inconsistently observed. Abnormalities have been reported in cortical lamination (Bailey et al., 1998; Weidenheim et al., 2001) and in neuronal density (Bailey et al., 1998). However, these findings have been largely inconspicuous in our serial section material. Brainstem malformations have been observed in the arcuate nuclei and inferior olive (Bailey et al., 1998; Weidenheim et al., 2001). In addition, decreased neurons in the facial nucleus and superior olive, as well as shortening of the distance between the trapezoid body and inferior olive, have been reported in one case (Rodier et al., 1996). In contrast, our studies showed enlarged olivary but plentiful neurons in the brainstem of all of the autistic patients less than 13 years of age and small pale neurons in adult autistic subjects. In addition, some of the inferior olivary neurons were noted to be clustered peripherally in all brains. More recently, we have begun to review systematically our brainstem material and have found one case in which there is an ectopic accumulation of neurons on the surface of the inferior cerebellar peduncle (Figure 9.1). These cells are in the position of the migratory stream of neurons that extends from a germinal zone in the rhombic lip of the forth ventricle to their definitive position in the inferior olive, arcuate nuclei, and basis pontis (Rakic, 1982). Further detailed analysis of this material is under way. Although not a consistent finding, the presence of cortical and brainstem malformations in some autistic brains indicates a pathology dating to the period of fetal development, possibly as early as the first trimester.

Conclusion

Although there has been some variation in the microscopic findings from brain to brain, some common themes are beginning to emerge. We noted, for example, that all of our autistic brains have shown reduced numbers of cerebellar Purkinje cells and the presence of small neuronal size and increased cell packing density in the entorhinal cortex and medially placed nuclei of the amygdala, regardless of age, sex, and functional ability.

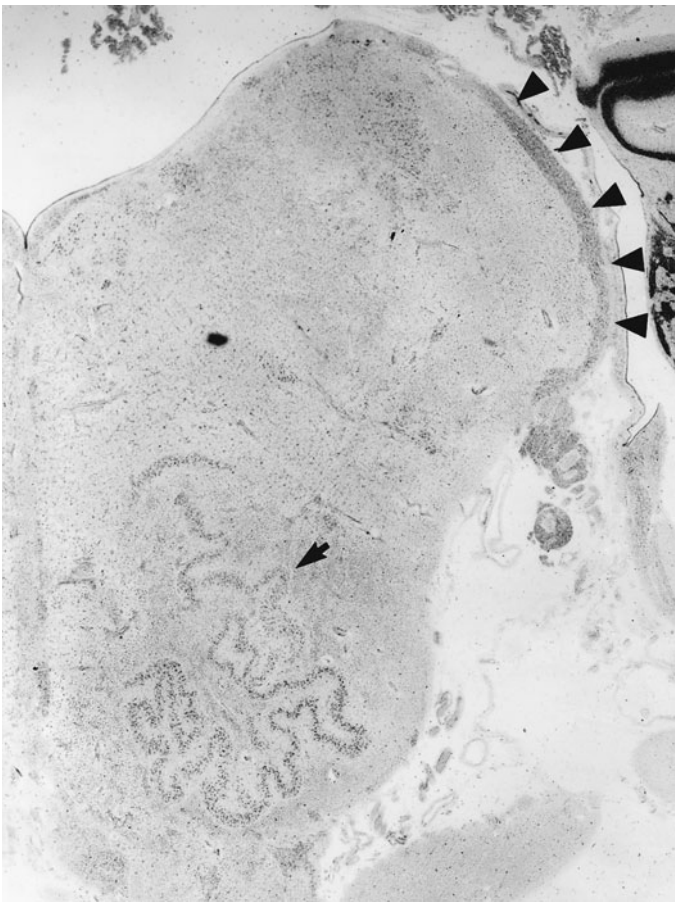

FIGURE 9.1. Macrophotograph of a Nissl-stained section from the medulla of a 9-year-old autistic boy to show neurons arrested in migration (arrowheads). These ectopic cells are in the position of a migratory stream of neurons that extends from a germinal zone in the rhombic lip of the fourth ventricle to their definitive position in the inferior olive, the arcuate nuclei, and basis pontis. Note the abnormally folded inferior olive (arrow), a finding also noted by Bailey et al. (1998).

Despite these common features, microscopic analysis of our own material and the findings of other investigators have revealed a broader spectrum of anatomic findings than originally appreciated. Although the limbic system and cerebellar circuits continue to show obvious and consistent abnormalities, additional observations include increased brain weight and volume in childhood, microscopic differences between children and adults, and some evidence of scattered malformations. The significance of these multiple, seemingly unrelated pathologies is uncertain,

but may be related to multiple genes that have been hypothesized to be at the basis of the ASD. Alternatively, or possibly in conjunction with genetic causes, there may be a single etiologic event that occurs very early, perhaps at conception, that initiates a process that impacts on multiple aspects of brain development.

Anatomic findings continue to support the notion that autism and its related disorders have their onset prenatally, and there is now mounting evidence to suggest that the process that underlies these disorders continues into adulthood. The observation of increasing brain weight and volume in early childhood is particularly intriguing, given some evidence that increased head circumference tends to become obvious at about 18 months of age, a time when many parents begin to recognize symptoms in their children. What this underlying process may be and how it affects brain development throughout life will be one of the most important and interesting questions for future research.

Additional neuroanatomic studies will be important to elucidate the relationship between microscopic structural abnormalities and clinical presentation, as well as to stimulate basic science research questions, which will aid in our understanding of autism. Neuroanatomic observations should also provide a "yardstick" to test etiologic hypotheses and to offer important leads for future genetic and neurochemical investigations, which may result in more accurate early identification and more effective medical and clinical interventions.

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10

The Brainstem in Autism

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Historically, investigators interested in the neurobiology of autism have focused their attention on the forebrain. There are symptoms of autism that suggest forebrain dysfunction, for example, deficits in executive function (e.g., Ozonoff et al., 1991). Most symptoms, however, cannot be localized to a particular brain region or system. For example, we know the cortical regions critical for speech, but we do not know any region that plays a role in pragmatic language. We know that injury to almost any part of the brain will affect social behavior (e.g., temporal lobe lesions [Kluver and Bucy, 1939], neonatal exposure to lead [Laughlin et al., 1991], prenatal exposure to methylmercury [Burbacher et al., 1990], maternal deprivation [Seay and Harlow, 1965]), but we do not know any region or system that supports the specific social deficits characteristic of autism. In addition, when brain structures appear to function abnormally in people with autism, as in the reported malfunction of the fusiform gyrus during facial recognition (Schultz et al., 2000), we have no idea whether the malfunctioning region is abnormal, or whether it is malfunctioning because of receiving abnormal input.

Over the past decade, evidence for brainstem injury in autism has emerged from a variety of sources. The discovery of several environmental factors that increase the risk of autism has provided a wealth of information and allowed the development of animal models. Anatomic studies have shown great variation across cases, but several histologic anomalies of brainstem structure have been reported. Behavioral study of ever simpler functions has demonstrated that people with autism spectrum disorder (ASD) have deficits much more basic than those described by the diagnostic symptoms. Finally, several developmental disorders characterized by brainstem anomalies have been revealed to have high rates of comorbidity with autism. This chapter attempts to make these varied lines of evidence accessible to readers interested in autism.

Exposures to Thalidomide, Valproic Acid, and Misoprostol

In 1991, Miller reported the results of an extensive study of dysmorphology and neurologic dysfunction in 86 Swedish adults exposed to thalidomide in utero. Three years later, Strömland and others (1994) reported that four of the 86 cases had autism. Even if one uses the highest reported prevalence numbers for ASD (6.3/1000; Chakrabarti and Fombonne, 2001), the data indicate that thalidomide increases the risk of autism more than sevenfold. The association is actually even more impressive than these data suggest. Using their detailed data on physical malformations and the known timing of thalidomide's effects, Miller and Strömland were able to pinpoint a critical period during development when all four of the people with autism had been injured. Each had ear malformations, which appear with injury as early as day 20 after conception, but not forelimb malformations, which begin with injury on day 24. Reviewing all cases in the study showed that only 15 had evidence of injury during that period. Thus, exposure to thalidomide during the critical period increased the risk of autism more than 40-fold.

The critical period identified in the thalidomide study, 20–24 days after conception, is when only a few neurons have begun to form. These are the motor neurons of the cranial nerve nuclei (Bayer et al., 1993). They control movements of the eyes, face, throat, jaw, and tongue. Because the investigators had studied their patients so intensively, they were able to report that four had abnormalities of eye movement involving the third and/or sixth cranial nerves; four had Moebius syndrome, characterized by dysfunction of the sixth and seventh cranial nerves; and two had abnormal lacrimation, which results from misdirected projections of the seventh cranial nerve. The neurologic problems observed in cases with autism after exposure to thalidomide are consistent with what we know about the developing brain, and they confirm the critical period deduced from physical malformations.

The thalidomide study provides the strongest possible evidence that brainstem injury plays a role in the initiation of autism subsequent to thalidomide exposure. It cannot answer the question of whether brainstem injury is present in other cases with other causes. However, even the literature at the time suggested that craniofacial anomalies and neurologic dysfunctions might be common in autism. For example, several articles had highlighted the minor craniofacial anomalies that are more frequent in people with autism than in controls (Steg and Rapoport, 1975; Walker, 1977). Abnormalities of eye movement had been reported in two studies (Rosenhall et al., 1988; Scharre and Creedon, 1992), and deficits in facial expression are part of the diagnostic criteria (American Psychiatric Association, 1994).

Expression of craniofacial physical abnormalities and neurologic deficits are not markers for autism, because some cases have neither, but they are common. In one

study, the most typical anomaly, posterior rotation of the ears, occurred in 42 percent of a population of children with autism from Nova Scotia (Rodier et al., 1997). In probands with ASD selected for evidence of familial etiology, 56 percent had some physical or neurologic anomaly (Ingram et al., 2000). In cases from another site in the United States, 42 percent had at least one example of dysmorphology, and 22 percent had three or more (Miles and Hillman, 2000). In cases ascertained through clinics in Sicily, 56 percent were dysmorphic (V. Romano, personal communication). A recent anthropometric study (Deutsch et al., 2002) adds several dysmorphic features to the list of those associated with autism. For example, the maxilla is wider than normal. Another interesting finding of this study was that children with a history of specific language impairment share some of the dysmorphologies characteristic of autism, such as macrocephaly and brachycephaly.

Thus, thalidomide-induced autism is not dissimilar to idiopathic autism with regard to the frequent presence of a specific set of malformations and neurologic dysfunctions. There is no doubt that many of these features can be induced only early in the first trimester, suggesting that many cases of autism arise from injury at this time.

Exposure to valproic acid in utero was recognized as a possible risk factor for autism in a series of case reports (Christianson et al., 1994; Williams and Hirsch, 1997; Williams et al., 2001), and then in a cohort study (Moore et al., 2000). Unlike the thalidomide data, which could be related to studies of brief exposures, all the valproic acid cases are assumed to have been exposed chronically. But even with no firm information on the critical period for induction of ASD, the odds ratio for the risk of these disorders in exposed individuals is about 17. Furthermore, their craniofacial malformations suggest very early injury.

Most recently, Miller and Ventura (2001) reported an increased risk of autism in children with Moebius sequence resulting from exposure to misoprostol in the sixth week after conception. We discuss the study in detail in a later section of this chapter, when Moebius sequence is discussed as a condition comorbid with autism. It is mentioned here as an example of a single teratogenic exposure at a known stage of development that increases the risk for autism. Although its critical period differs slightly from those of thalidomide and valproic acid, the data indicate that misoprostol, too, causes an early injury to the brainstem, exemplified by dysfunction of the sixth and seventh cranial nerves.

Morphologic Studies

Many histologic studies of the brains of people with ASD have focused on forebrain structures. There are reports of both negative and positive results, and these

are discussed in more detail in other chapters of this volume. Here, we describe anomalies of the brainstem and the intimately related cerebellum.

A number of anomalies of the brainstem have been reported in individual cases. For example, one of the six brains described by Bailey and others (1998) had an extra tract running through the pontine tegmentum. Another had pyramidal tracts that were almost fused in the midline. Two brains had enlarged arcuate nuclei in the medulla, two had loosely grouped neurons in the locus coeruleus, and one had a small midbrain. A brain examined by Rodier and others (1996) had a major reduction of neuron numbers in the facial nucleus and no structure corresponding to the superior olive. Measurements of serial sections demonstrated that the brainstem was shortened in the region derived from the fifth rhombomere, with caudal structures, such as the hypoglossal nucleus and the inferior olive, shifted orally.

Two groups of investigators have observed that the inferior olive is abnormal in many cases of autism. Kemper and Bauman (1993) reported that the neurons appeared to be larger than normal in the brains of young people with autism and smaller than normal in adults with autism. In five of six of the brains examined, the investigators also noted abnormalities in the position of the olivary neurons within the convolutions of the olive.

In the brains examined by Bailey and others (1998), three of five with adequate sections had abnormal structure of the inferior olive. One had bilateral anomalies, with the normal ribbon of tissue broken up into short segments, and what appeared to be an extra medial accessory olive. Two other brains had bilateral single breaks in the continuity of the olive. All three of these brains had ectopic neurons lateral to the olive, or in the inferior cerebellar peduncle, as did two brains with normal olivary architecture.

The deep nuclei of the cerebellum have also been examined in several studies. Bauman and Kemper (1994) found the fastigial, globose, and emboliform nuclei, but not the dentate nucleus, to have reduced cell numbers in adult cases of autism. Bailey and others (1998) noted breaks in the ribbon of neurons that forms the dentate nucleus in two of their six cases.

Three groups have reported reductions of Purkinje cell numbers in autism (Bauman and Kemper, 1985, 1994; Ritvo et al., 1986; Bailey et al., 1998). Counts of neuron numbers in sample areas revealed decreased density of Purkinje cells in the brains of people with autism compared to the brains of controls. Although the deficit in Purkinje cell density is the most consistent finding in brains of people with ASD, the finding is not without controversy. First, the density of neurons is rarely altered when neurons fail to form (discussed in Rodier and Reynolds, 1977). Reduced density is a characteristic of conditions in which cells

establish their positions and then die. Thus, the reported changes in density suggest that Purkinje loss in autism may not occur in the embryonic period when these cells form. However, we know that Purkinje cell death after 30 weeks of gestation is reflected in massive cell death in the inferior olive (Takashima, 1982). That such neuron loss has not been observed in the olive implies that the Purkinje cells were lost before 30 weeks. However, there are some indications that Purkinje cells may continue to succumb to injury in children and adults with autism. Gliosis in the cerebellum was observed in several of the cases in Bailey and colleagues' study (1998), suggesting that some of the loss may be due to ongoing problems, such as seizures, rather than to developmental reorganization of the brain.

Although the cerebellar results are ambiguous with regard to the time of injury, the disparate anomalies of the brainstem are strong evidence for abnormal development in early embryonic life. For example, we know that in one case the facial nucleus failed to form, rather that losing neurons after their formation, because the capsule of fibers that outlines the normal structure was not present, as it would be if the nucleus had been established and then lost its neurons (Rodier et al., 1996). Similarly, because the basic tracts running along the neuroaxis are present very early (e.g., Muller and O'Rahilly, 1988), the extra tract reported by Bailey's group (1998) could not have formed late in development.

The importance of anatomic evidence is often difficult to judge, because anomalies of structure may or may not result in functional consequences. Furthermore, because autism is thought to have multiple etiologies, there is no reason to think that any anatomic alteration should characterize all cases. What makes brainstem effects important is that they indicate injury before the forebrain structures form. Unless repeated injuries are required to initiate the developmental alterations that underlie ASD, brainstem effects suggest a scenario in which forebrain anomalies are sequelae of brainstem injury.

A series of studies in rats demonstrates how some of the anatomic features reported in autism might arise. Exposing rats to valproic acid during neural tube closure models an exposure that is known to increase the risk of autism in humans (Moore et al., 2000). The exposure reduced body weight and brain weight only slightly, but had major effects on some early-forming parts of the brain. For example, neuron numbers in cranial nerve motor nuclei, such as the abducens, trigeminal, and oculomotor, were significantly reduced (Rodier et al., 1996). The inferior olive forms at the same stage of development as the motor nuclei (Bayer et al., 1993), and the total number of neurons in that structure was reduced by almost 50 percent in the valproate model (Rodier and LaPoint, 2001). Because exposure was designed to precede the earliest neuron production for the Purkinje cells, the status of the cerebellum would answer the question, "Can

Purkinje cell numbers be reduced as a secondary effect of brainstem injury?" Ingram and others (2000) found that the volume of the cerebellar cortex and the number of Purkinje cells were significantly reduced overall in the valproate model. Furthermore, the greatest deficiencies were in the posterior vermis, a region reported to be reduced in some imaging studies of autism (e.g., Courchesne et al., 1994). These effects parallel many of those reported in human cases, but are not identical. For example, Purkinje cell density was normal in the animal model; only total cell number and tissue volume were significantly reduced by valproate exposure. The human histologic studies described above did not evaluate total neuron number or tissue volume.

Studies of Simple Behaviors

In the past 10 years, psychologists have begun to search for behavioral differences that distinguish people with ASD and that can be related to neural systems. The behavior for which the contributing neural pathways are known best is eyeblink conditioning. The classical conditioning paradigm that pairs a tone with aversive stimulation of the eye, such as an air puff, has been studied in many animal species and in several conditions of human neurologic dysfunction (e.g., mental retardation [Orlich and Ross, 1968]), Alzheimer disease [Solomon et al., 1991], epilepsy [Daum et al., 1991]). Sears and colleagues (1994) compared eyeblink conditioning in individuals with autism and IQ-matched controls. They found that people with autism developed the conditioned response more rapidly than did controls, and that they exhibited abnormal timing and amplitude of the conditional blink response. These results have been replicated in children with ASD (Herbert et al., unpublished data). The new study included many control conditions to be sure that the effects were not a result of purely sensory or motor anomalies. For example, the data show that children with autism are not more sensitive to the tone or the air puff than are controls.

The pathway required for the acquisition of the conditioned association and response timing and amplitude has been described (summarized by Mauk and Ruiz, 1992). It consists entirely of brainstem–cerebellum–brainstem loops. The unconditional stimulus (the air puff) activates climbing fibers projecting from the inferior olive to the cerebellar cortex (Mauk et al., 1986). The tone (the conditional stimulus) activates mossy fibers projecting to the cerebellar cortex (Steinmetz et al., 1986). Each pathway sends collateral fibers to the nucleus interpositus. The output of the cerebellar cortex is through the Purkinje cells' projections to the nucleus interpositus (the rodent and rabbit equivalent of the human globose and emboliform nuclei). The projections of the nucleus interpositus go to the red nucleus and are distributed to the facial nucleus by way of the rubro-

bulbar tract. Conditioning does not occur in the absence of the nucleus interpositus (Steinmetz et al., 1992). Lesioning the cerebellar cortex greatly retards the acquisition of the association, but does not eliminate it (Lavond and Steinmetz, 1989). The final motor response is eliminated in the contralateral eye by lesions of the red nucleus, but the association apparatus is intact after this lesion, because the ipsilateral eye exhibits normal conditional responses (Rosenfield et al., 1985). The eyeblink results in people with autism indicate a reorganization in the pathway that is probably centered in the cerebellar cortex.

Because the eyeblink conditioning paradigm can be duplicated in animals, it has been possible to test valproic acid–exposed rats to determine whether their behavior parallels that seen in people with ASD. Stanton and colleagues (2001) reported the same rapid acquisition and response timing anomalies observed in human cases of ASD in rats exposed to valproic acid during neural tube closure. This observation is, by far, the most specific behavioral parallelism ever discovered in an animal model of autism.

Another example of a simple behavior that is abnormal in autism is visual orientation. Bryson and colleagues began with complex attention tasks, but eventually focused on reflexive orientation as an example of a very basic behavioral disturbance characteristic of children with ASD (Bryson et al., in press; Landry and Bryson, in press). As they face a display, the subjects naturally orient to a stimulus of flashing colored lights. On a "shift" trial, the lights go off and then reappear in another part of the visual field. All subjects shift their orientation to the new stimulus. On a "disengage" trial, the first stimulus stays on while the second appears. Typically developing subjects reorient to the second stimulus almost as rapidly as on a "shift" trial, but those with ASD perform very differently. The latter frequently reorient to the new stimulus slowly or fail to reorient at all. It is as though they are "stuck" on the first stimulus. Whereas their shift data parallel those of controls, they have difficulty disengaging. The ability to disengage reliably develops between the second and fourth months in infants (Johnson et al., 1991), and children with various brain damage syndromes, such as Down syndrome, exhibit normal disengagement (Landry and Bryson, in press). The deficiency in the disengagement operation appears to be specific to ASD. The pathways involved in this simple behavior are not known, but the early development of the ability to disengage and the lack of deficits on this task in other neurologic disorders are consistent with a brainstem mechanism, such as disruption of reflex pathways in the superior colliculus.

It has been noted for many years that people with autism have reduced or abnormal facial expression compared to controls. This feature has been described as reflecting a deficit in social or affective development. However, there

is evidence that at least part of the underlying cause is a deficit in motor function (Rogers et al., 1996). Using videotapes of children in play situations, Czapinski and Bryson (2003) evaluated the percentage of time spent exhibiting facial expressions recognizable to observers (e.g., surprise, neutral, negative). Compared to children with typical development or those with language delay, children with ASD spent significantly less time showing enjoyment and significantly more time showing neutral or negative expressions. Atypical expressions, including unilateral facial movements, were significantly more common in children with autism.

Applying the coding system of Izard (1971) to videotapes revealed another important difference between children with ASD and others. Czapinski and Bryson (in press) found that movements of the brow did not differ between groups, but movements of the mouth region and the muscles around the eyes were much less frequent in children with autism than in those with language delay or typical development. This pattern, in which the three branches of the facial nerve display different levels of dysfunction, is known as supranuclear palsy. It implies a neurologic deficit in higher-order structures controlling the facial nucleus. In contrast, some of the atypical expressions observed implicate dysfunction of the brainstem nucleus itself. Thus, studies of facial expression suggest that the motor control of facial movement is dysfunctional at several levels of the neuroaxis in people with autism.

A particularly striking set of findings regarding motor function in autism comes from studies of speech and motor development. Using interviews with caregivers and analyses of home videos, Gernsbacher and colleagues (2002) found that a subset of children with ASD are distinguished by failure to exhibit a number of motor actions at the stages when they typically appear. For example, at 6 months, most children "blow raspberries" and grasp at dangling earrings. At 24 months, most can turn a round doorknob and suck liquids through a straw. Those children with the greatest delays in oromotor and manual-motor development in early life developed little or no speech later in childhood. Within the autism sample, children with the highest and lowest scores in the retrospective study of motor development markers were compared on the Praxis Test of the Boston Diagnostic Aphasia Exam and the Kaufman Speech Praxis Test for Children. Children identified by poor motor development were severely impaired on these tests and had minimal current-day speech. Interestingly, despite their problems with speech, some of the minimally verbal children had good receptive language. Thus, it appears that the problems with speech in some children with ASD may not be related to the problems with language that characterize the diagnostic category. Rather, the speech problems may stem from apraxia, whereas the language problems stem from cognitive dysfunctions.

The neurobiologic sources of oromotor apraxias are not well defined, but there is good evidence that lesions of the frontal cortex can induce the symptoms (e.g., see the review by Paillard, 1982). However, it is known that surgery for tumors of the posterior fossa carries a substantial risk for oral pharyngeal apraxia and mutism (Dailey et al., 1995), and the critical region for the effect is located in the inferior vermis of the cerebellum. In addition, impairment of speech is a feature of familial olivopontocerebellar degeneration, as described by Koeppen and Hans (1976).

Simple behaviors are not the only ones affected in autism. But they indicate that the neurologic deficits of the disorder are not restricted to cognitive processes. Rather, some of the deficits appear to be much more basic in nature.

Comorbid Conditions

A number of conditions comorbid with autism involve widespread or unspecified changes in the brain. For example, phenylketonuria, tuberous sclerosis, and fragile X syndrome are conditions that often exhibit behaviors diagnostic of ASD, but they are not characterized by definitive, localized lesions that would help us understand the neurobiology of autism. The CHARGE association and Goldenhar syndrome are congenital conditions with multiple craniofacial anomalies. In each, some cases meet the criteria for ASD (Landgren et al., 1992; Fernell et al., 1999). These genetic disorders reinforce the idea that early injuries are important in at least some cases of autism, but the morphology of the brains has not been described. There are comorbid conditions, however, that are instructive regarding the neuroanatomy and embryologic timing of initiating events in autism.

Joubert syndrome (Joubert et al., 1969) is a rare recessive genetic disorder that appears to be caused by different alleles in different families (Saar et al., 1999). The victims have minor craniofacial anomalies, but their main problems are neurologic. At birth, they exhibit brainstem dysfunctions, including breathing problems, abnormal eye movements, poor sucking, and lack of control of the tongue, and as they grow up, all cases are seen to have mental retardation (Maria et al., 1999a). Imaging and histology of the brains of people with Joubert syndrome indicate that they have agenesis of the cerebellar vermis and the motor nuclei of the brainstem tegmentum (Maria et al., 1999b; Yachnis and Rorke, 1999). In addition, the brain described by Yachnis and Rorke had a brainstem shortened at the junction of the pons and midbrain. The caudal medulla and inferior olive were dysplastic, and the superior cerebellar peduncles were uncrossed. In the forebrain, some subcortical structures were abnormal. For

example, the posterior thalamus was fragmented and the subthalamic nucleus could not be identified (Yachnis and Rorke, 1999).

Holroyd and others (1991) reported that some cases of Joubert syndrome had symptoms of autism. Ozonoff and others (1999) tested 11 cases and found that four met the ADI/ADOS (autism diagnostic interview/autism diagnostic observation schedule) criteria for autism and all had symptoms of ASD.

The pathology of Joubert syndrome is much more extensive than anything observed in cases of idiopathic autism, but the findings overlap with many of the features reported by investigators of autism. This supports the hypothesis that cerebellar–brainstem effects may be important in the functional abnormalities diagnostic of ASD.

Gillberg and Steffenberg (1989) were the first to notice that autism was comorbid in some cases of Moebius syndrome—a congenital dysfunction of the innervation of the muscles of facial expression and the lateral rectus of the eye. They evaluated a larger sample recruited from residential facilities (Miller and Strömland, 1999; Johansson et al., 2001). The rate of ASD diagnosis was 26 percent. In a parallel study in Canada, cases have been recruited from international meetings of Moebius families and from families seeking surgical intervention. The rate of ASD diagnosis in this group is at least 30 percent (McConnell et al., 2002). All the Moebius cases comorbid for ASD studied in Sweden were cognitively impaired. However, one-third of the cases studied in Canada had IQs within the average or above-average range. Thus, the association of Moebius syndrome with autism is not dependent on the presence of mental retardation. Instead, it suggests that early injury to the brainstem in the region of the pontomedullary junction increases the risk of ASD.

Because Moebius syndrome is sometimes genetic and sometimes environmentally caused, it has been possible to investigate the connection between the syndrome and autism in cases with different causes. Moebius syndrome is one of the birth defects that result from failed abortions attempted with the prostaglandin misoprostol (Gonzalez et al., 1993, 1998). Miller and Ventura (2001) compared the rate of autism in children with idiopathic Moebius syndrome to the rate in children with Moebius syndrome subsequent to exposure to misoprostol in utero. Three of 15 idiopathic cases had autism and four of 15 cases exposed to misoprostol had autism.

This one small study answers several important questions about the role of brainstem injury in autism. It confirms that very different mechanisms of injury can lead to developmental effects sufficient to increase the risk of autism. Little is known about the mechanisms by which thalidomide, valproic acid, and ethanol injure the nervous system, but all are known to interfere with cell pro-

duction. In contrast, misoprostol is thought to act by causing transient ischemia, killing cells with a tenuous blood supply. Misoprostol is almost invariably taken in the sixth week after conception, whereas the critical period for the other environmental risk factors is earlier. Thus, the study suggests that there must be a range of critical periods when brainstem injury can increase the risk of autism. That the risk of autism is increased in Moebius syndrome, regardless of its cause, focuses attention on the location of the injury as the common feature shared by all Moebius cases with autism.

In summary, there is ample evidence that brainstem injury plays a role in many cases of ASD. Minor malformations that can only arise in the early embryo are seen in substantial numbers of people with autism. Some environmental factors that increase the risk of autism have been shown to have their critical periods at times when only brainstem neurons are forming. Human histology has revealed oddities of the brainstem and cerebellum in some cases of autism, and changes in a few structures, such as the inferior olive and cerebellum, seem to be present in most cases. More and more behaviors mediated by the brain stem or cerebellum are being found to be abnormal in autism. A number of genetic and environmental syndromes in which the risk of autism is elevated are characterized by brain stem pathology.

The studies cited do not argue against pathology or dysfunction in other parts of the brain in autism. On the contrary, it seems likely that many parts of the nervous system in ASD are abnormal. However, there is no region but the brainstem for which so many lines of evidence indicate a role in autism.

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11

Myelin and Autism

Omanand Koul, Ph.D.

White matter consists mainly of myelin, a multilayered membrane wrapped around axons. In comparison to other membranes in the cell, myelin is relatively rich in lipids and low in protein. Thus, myelin appears white in comparison to the surrounding neuron-enriched areas, which appear gray. Myelin is essential for efficient conduction of electrical impulses and for structural integrity of axons. Disorders or insults that cause dysmyelination and/or demyelination lead to alterations in transmission of action potentials and may also lead to axonal degeneration, and thus, interfere in the impulse-driven activation of post-synaptic stage-specific events during development of the central nervous system (CNS). Therefore, alterations in myelin can lead to additional secondary functional abnormalities of the nervous system.

The pioneering neuropathologic studies on the alterations of the brain in autistic individuals by Bauman and Kemper (1985, 1994) laid the foundation for later investigations that used neurophysiologic, immunologic, and magnetic resonance techniques. These studies support the possibility that alterations in myelin may be one of the correlates of this disorder. For example, brainstem auditory-evoked potentials in autistic children have significantly increased transmission times (Wong and Wong, 1991). Such observations have led investigators to support the hypothesis of a maturational defect in myelination (McClelland et al., 1992) and to explain the reduction in brainstem size observed on magnetic resonance imaging (MRI) (Miyazaki and Hashimoto, 1991; Hashimoto et al., 1992). Similarly, a possible involvement of myelin is indicated by reports that antibodies against myelin basic protein (MBP) circulate in the plasma of autistic patients (Singh et al., 1993).

The results of an elaborate MRI investigation of autistic individuals of various age groups by Courchesne and colleagues have added the feature of larger volume of white matter on the list of the hallmarks of autism (Courchesne et al., 2001). Their data indicate that white matter occupies abnormally large volumes

in cortical and cerebellar regions of the brain in younger patients. They studied 60 autistic individuals 2–16 years old and compared them to age-matched controls. Thirty of the subjects were diagnosed and scanned at 5 years of age. The other 30 were scanned at 2–4 years of age and diagnosed 2.5 years later. The researchers measured volumes of intracranial space, whole brain, gray matter, white matter, and cerebrospinal fluid (CSF). The neonatal brain volume of autistic individuals was deemed to be within normal range; however, for children in the age group of 2–4 years, the brain volume was larger than normal in 90 percent of boys. The cerebral white matter was larger by 18 percent in 2- to 3-year-olds. The cerebellar white matter volume was even greater—by 39 percent. However, in adolescents, there were no significant differences in the volume of white matter between normal and autistic individuals. Of added interest is the observation that, in comparison to controls, the rate of growth of white matter in autistic individuals slows down after the initial burst of activity and thus does not keep up with the overall rate of brain growth. The data support the hypothesis that the initially larger volumes of white matter may be due to a faster rate of biosynthesis (of immature myelin) disproportionate to the normal rate of compaction. In addition the immature myelin may also lack specific components otherwise needed for normal compaction. Similar imaging studies by Dr. Martha Herbert also indicate alterations in volume of white matter in autism (Herbert, personal communication).

Other early imaging studies, although not as comprehensive, also suggested alterations in brain and myelin volume (Filipek et al., 1994; Piven et al., 1997a, 1997b). Furthermore, results from ^{31}P magnetic resonance spectroscopy indicated the possibility of altered metabolism of phosphate-containing compounds in high-functioning autistic adolescent and adult men (12–36 years of age) (Minshew et al., 1993).

Imaging studies of the corpus callosum indicate its volume to be disproportionately low in autistic subjects relative to cerebral cortical and white matter volume (Minshew and Dombrowski, 1994; Egaas et al., 1995). In a different study, the nuclear magnetic resonance (NMR) signal intensity from five regions of the midsagittal corpus callosum in autistic patients also indicated callosal narrowing compared with normal controls, but this was attributed to a generalized loss of axons rather than to the absence of myelin (Belmonte et al., 1995). However, because axonal loss could be secondary to myelin defects, this attribution still leaves the data open to interpretation. Other imaging analyses have also indicated a thinning of the corpus callosum in a subset of autistic individuals, in addition to a loss of white matter in parietal lobes in some autistic patients (Courchesne et al., 1993). More recent studies indicate that only the anterior subregions are thinner in autistic individuals (Hardan et al., 2000).

Although white and gray matter in living tissues can be visualized and demarcated by MRI, the technique lacks the resolution necessary to identify alterations at the level of individual molecular components. Therefore, molecular analysis of white matter could eventually help in interpreting the data from the MRI. Because corpus callosum is rich in myelin, we have chosen it for our investigation.

Maturation of Myelin

During development of the CNS, myelin is synthesized by oligodendroglial cells around the axons. Thus, appropriate signaling and interaction are necessary for the subsequent synthesis of biochemical components and assembly of mature myelin membranes. Failure to generate signaling cues and/or cell-cell interactions can alter the synthesis of appropriate biochemical components and lead to improperly synthesized myelin membrane. The process of myelination is susceptible to changes in the levels of hormones, environmental insults, and nutrition. For example, thryoxine and corticosteroids alter the course of myelination, and heavy metals inhibit the enzymes needed for some of the components of myelin biosynthesis (Grundt and Neskovic, 1985; Jungalwala et al., 1985; Domanska-Janik and Bourre, 1987).

In humans, biosynthesis of myelin starts during fetal life and continues postnatally for at least two decades. However, the initiation of myelination and its rate of formation are region specific (Yakovlev and Lecours, 1967; Kinney et al., 1988). The process includes stage-specific synthesis of membrane proteins and lipids to form myelin membrane (Koul et al., 1980, 1988; Koul and Jungalwala, 1981, 1986). The process is temporally and spatially modulated such that the relative rates of various components differ from one another to match the synthetic needs of the membrane. Although the components have a major structural role, some may also be involved in active processes, such as biosynthesis and degradation, signal transduction, and apoptosis (Koul et al., 1980; Lees and Bizzozero, 1992).

The major lipids in myelin include glycolipids, phospholipids, and cholesterol; major proteins include proteolipid protein (PLP) and MBP; and minor proteins include myelin-associated glycoprotein (MAG) and Wolfgram proteins. Myelin resident proteins also include enzymes involved in various functions, including biosynthesis and degradation (Ledeen, 1992). The current thinking is that the bulk of the myelin membrane is synthesized largely by the activity of enzymes in nonmyelin compartments of the oligodendroglial cells, and the growing myelin membrane is loosely wrapped around axons. Compaction of the loosely wrapped membranes involves loss of fluids trapped between the layers of membrane and adjustment of the lipid:protein ratio to match that present

in the adult myelin. Thus, the presence of abnormal myelin in autistic individuals could be due to an abnormality of any of the steps involved in the synthetic, degradative, or compaction processes. Thus far, brain MRIs of autistic individuals and our analytical data on components of postmortem brain tissue indicate the possibility that both the net rate of deposition of certain myelin components and the process of myelin compaction are altered in autism. The alteration in the rate of net deposition may be a result of an imbalance between the activity of synthetic and degradative enzymes, whereas the abnormality of the compaction process may be due to alterations of the enzymes involved in the flux of ions and water (e.g., carbonic anhydrase, sodium-potassium ATPase).

Goals of Tissue Research

Investigation of appropriately frozen brain tissue offers a unique opportunity to formulate and reconstruct the molecular signature of developmental events that lead to autism. The goal is to decipher the signature profile such that possible trigger points in metabolic pathways are identified in designing strategies for intervention for a better clinical outcome.

DRY WEIGHT AND WATER CONTENT

Known amounts of wet tissues were lyophilized for 2 days and immediately weighed. On a wet weight basis, about 70 percent of this tissue is water and the rest (~30%) is made up of solids, which include lipids and proteins. Consistent with published reports about white matter, total proteins constitute ~30–40 percent of the dry weight (~12% of wet weight) and the rest includes lipids and salts (Figures 11.1, 11.2). Surprisingly, there were no significant differences in the percentages of dry weight and water content of the corpus callosum between autism and controls. In view of the recent MRI data obtained with adolescent autistic individuals (by Egaas et al., 1995), any significant differences in these parameters in adult tissue would not be expected. However, studies with tissues from younger individuals may indicate otherwise.

PROTEIN

Total protein was determined in tissues by the Pierce BCA assay. Irrespective of the source, the total protein content of the corpus callosum was between 30 and 40 percent of the total dry weight. The protein profile of corpus callosum is dominated by the major myelin-specific proteins, including PLP and MBP. However, no major differences are apparent in these proteins between autism and controls.

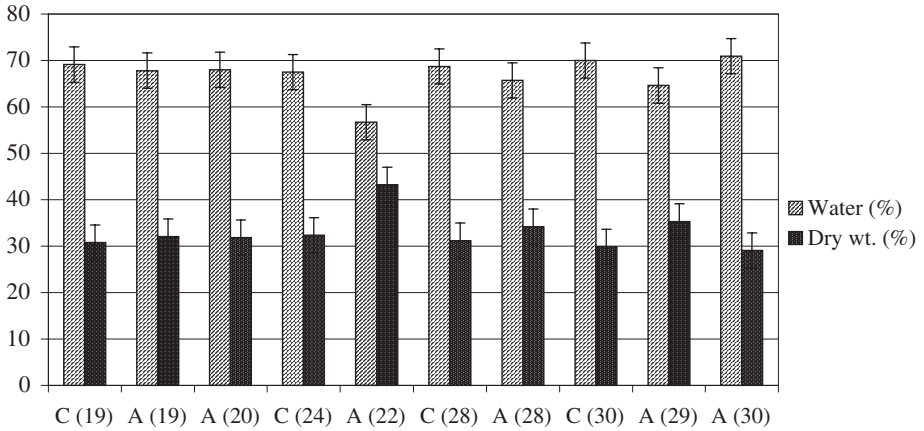

FIGURE 11.1. Water content of the corpus callosum. Known amounts of tissues were lyophilized for 48 hours and weighed immediately to determine the loss of water. The ages of the subjects in years are given in parentheses. A, autistic subject; C, control.

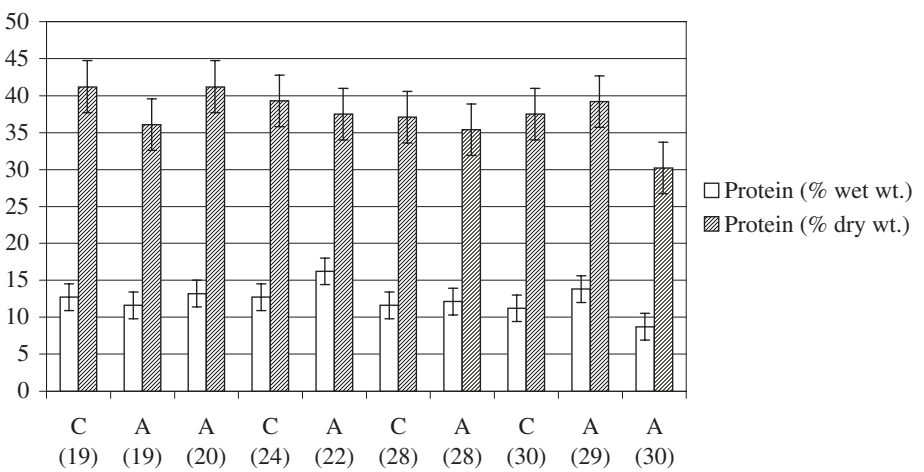

FIGURE 11.2. Protein content of the corpus callosum. Protein was determined in aliquots of aqueous tissue homogenates by the Pierce BCA method, and the data expressed as a percentage of the original wet and dry weights. The ages of the subjects in years are given in parentheses. A, autistic subject; C, control.

Additional proteomic techniques are being employed to explore this aspect in more detail.

CYCLIC NUCLEOTIDE PHOSPHOHYDROLASE

The enzyme 2′,3′ cyclic nucleotide phosphohydrolase is enriched in myelin and is used as a myelin marker. In some disorders that involve myelin degeneration, the activity of this enzyme is altered. We assayed the activity in homogenates (Koul et al., 1980) and observed no significant differences between controls and autism. The data suggest that myelin in the adult is not degenerated in autism.

LIPIDS

Lipids were obtained by a modification of the method of Folch et al. (1957). Total lipids were subfractionated and separated into neutral, glycolipid, and phospholipid fractions (Ledeen and Yu, 1982). Neutral lipids were chromatographed on a high-performance thin-layer chromatography (HPTLC) plate to separate cholesterol from its ester. Glycolipids were separated into neutral and acidic fractions on a diethylaminoethyl dextran (DEAE) Sephadex® column. The neutral glycolipids and gangliosides were each separated by HPTLC (Koul et al., 1980) to obtain individual components. Phospholipids were separated by two-dimensional HPTLC (Bodennec et al., 2000). Spots were visualized by iodine stain, charring with methanolic sulfuric acid or lipid-specific spray. The plates were scanned and densities compared.

CHOLESTEROL AND CHOLESTEROL ESTER

Total cholesterol and cholesterol ester were determined chemically and by HPTLC (Koul et al., 1980; Bodennec et al., 2000). There were no significant differences in the amounts of cholesterol in autism and controls. In addition, no cholesterol ester was detectable. The presence of cholesterol esters, an indicator of gross myelin pathology, has been reported in disorders that affect myelin.

GLYCOLIPIDS (NEUTRAL)

The neutral glycolipids in normal myelin are mostly composed of cerebrosides and ceramide dihexoside. Both these glycolipids are present in autism (Figure 11.3). However, some of the major differences are seen in the distribution of cerebrosides. In one subset of tissue from three autistic individuals, the total amount of cerebroside is reduced by 11 percent in comparison to control. The difference is more striking when the ratio of molecular species of cerebrosides is taken into

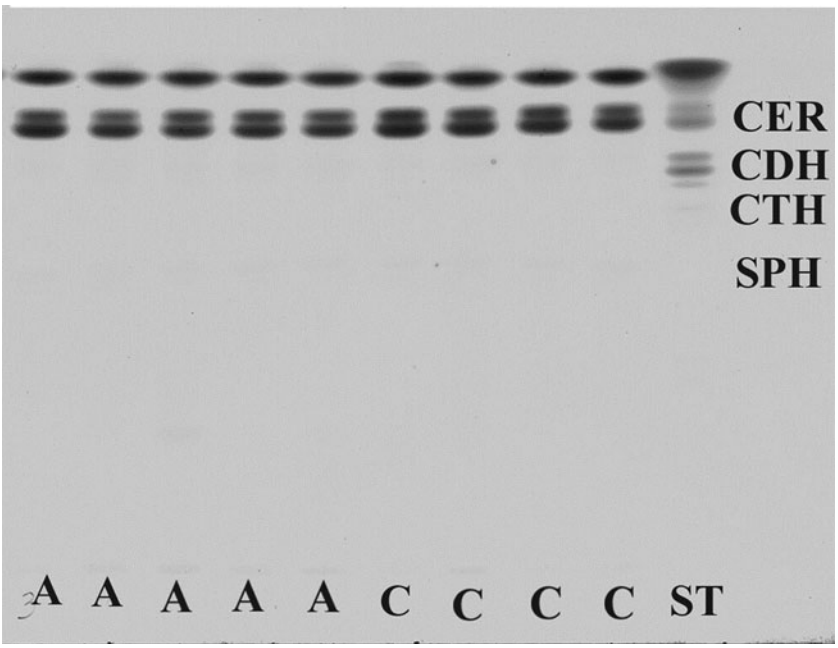

FIGURE 11.3. Neutral glycolipid profile in the corpus callosum (all adults). Equivalent tissue dry weights were spotted on HPTLC plate and developed in chloroform: methanol: aq Ca Cl2 (50:40:10, v/v). The plates were stained with iodine. A, autistic subject; C, control; CDH, ceramide dihexoside; CER, cerebroside; CTH, ceramide trihexoside; SPH, sphingomyelin; ST, standards.

consideration. In this subset, the ratio of nonhydroxy fatty acid–containing cerebrosides to hydroxy fatty acid–containing cerebrosides is only 0.62. The control value is 0.75. This finding suggests that most of the difference is due to a decrease in the expression of nonhydroxy fatty acid–containing cerebroside. A similar specific loss of nonhydroxy fatty acid–containing cerebrosides in white matter of some patients with multiple sclerosis has also been reported (Ohler et al., 2001). Significantly, isolated nonhydroxy fatty acid–containing cerebroside from myelin has been shown, by atomic force microscopy, to form nanotubes that aggregate with strong forces (Ohler et al., 2001). This observation led these investigators to suggest that nonhydroxy fatty acid–containing cerebrosides in myelin have a specific role in maintaining membrane curvature, adhesion, compaction, and stability (Ohler et al., 2001). Thus, a decrease in nonhydroxy fatty acid–containing cerebrosides may be a contributing factor in the formation of loose, uncompacted myelin in autism. In the second subset of tissues from autistic individuals, the ratio of nonhydroxy fatty acid–containing cerebrosides to hydroxy fatty acid–containing cerebrosides is 0.70, higher than the abovemen-

tioned subset but still lower than the control value of 0.75. This difference between the two subsets could either be due to the inherent heterogeneity of autism phenotype or to differences in sampled areas of the corpus callosum (see Hardan et al., 2000).

GANGLIOSIDES

Gangliosides are acidic glycolipids that are involved in membrane structure, calcium binding, cell adhesion, and signal transduction (Miljan et al., 2002). They have been shown to negate MAG-induced neurite outgrowth inhibition (Vyas et al., 2002). An association between specific gangliosides and cholinergic systems has also been put forward. The general profile of gangliosides in autism is similar to that observed in the control. The gangliosides include GM4, GM1, GD1a, GD1b, and GT (Figure 11.4). However, there is a significant reduction in gangliosides that are enriched in myelin (GM1), or specific to myelin (GM4). The gangliosde GM1 is reduced by about 11 percent in one subset of individuals and 26 percent in the second subset of autistic individuals. The myelin-specific ganglioside GM4 is decreased by 13 percent in one subset and by 38 percent in the second subset. The decrease in GM1 and GM4 in autism may either be due to a slower rate of biosynthesis or a faster rate of their degradation. It is known that during myelination, GM1 enters myelin early, followed by GM4 (Cochran et al., 1982). Thus, the loss of GM1 in white matter in autism may suggest a lesion early

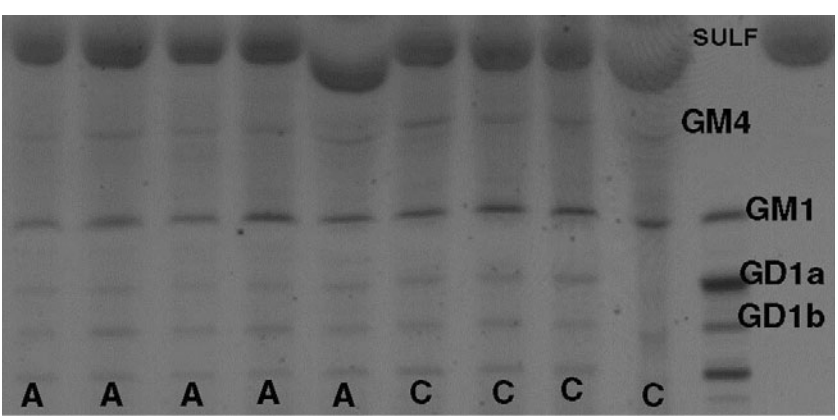

FIGURE 11.4. Ganglioside profile in the corpus callosum (all adults). Equivalent tissue dry weights were spotted on HPTLC plate and developed in chloroform: methanol:aq Ca Cl2 (50:40:10, v/v). The plates were sprayed with resorcinol. A, autistic subject; C, control; ST, standards; GD1a and GD1b, disialosylgangliosides; GM1 and GM4, monosialosylgangliosides; GT1b, a trisialosylganglioside; SULF, sulfatide.

during development and differentiation of oligodendroglia after the biosynthesis of cholesterol has occurred. In addition, because GM4 is a product of cerebrosides, a common defect in the pathway of cerebroside-GM4 biosynthesis is indicated. A report by Lekman et al. (1995) indicated an increase in GM1 content in the CSF of autistic individuals. They suggest that the increase in GM1 content was due to increased synaptic activity in autism. However, this would be possible only if the GM1 were released from neuronal membranes. However, if the GM1 were originating from oligodendroglia, then trafficking and targeting of GM1 may be at fault. Clearly, additional investigations are needed to clarify the process.

PHOSPHOLIPIDS

Phospholipids in two autistic individuals were analyzed and compared to their age-matched controls. The major phospholipids phosphatidyl choline (PC), sphingomyelin (SPH), phosphatidyl serine (PS), phosphatidyl inositol (PI), and phosphatidyl ethanolamine (PE) are present. The major differences are observed in the amounts of PE. In autistic individuals, the amount of PE is decreased by 1.5–2.5-fold (Figure 11.5). PE is located in the inner leaflet of the membrane. It can

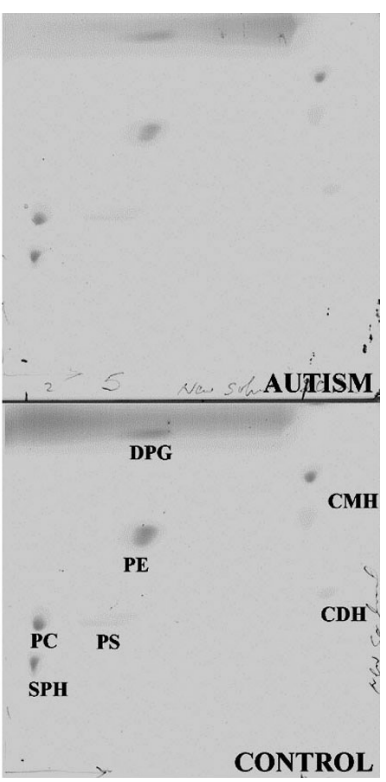

FIGURE 11.5. Two-dimensional separation of most phospholipids on an HPTLC plate. Equal amounts of dry weight equivalents were spotted for autism and control samples. The lipids were separated according to our method (Bodennec et al., 2000). Note the decrease in PE in autism. CDH, ceramide dihexoside; CMH, ceramide monohexoside; DPG, diphosphatidylglyceride; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; SPH, sphingomyelin.

flip-flop between the inner and outer leaflet. A specific aminophospholipid translocase is responsible for this phenomenon. The recent reports of the maternally imprinted gene, *ATP10C,* which encodes an aminophopholipid translocase as a candidate gene in Asperger syndrome (Meguro et al., 2001), makes this observation an important clue to be investigated in further detail. In addition, the role of phosphatidyl ethanolamine binding protein (PEBP) in autism needs to be investigated as well. The roles played by the translocase and PEBP may be interrelated.

Future Studies

HIPPOCAMPAL CHOLINERGIC NEUROSTIMULATING PEPTIDE AND AUTISM

Hippocampal cholinergic neurostimulating peptide (HCNP) promotes the development of cholinergic systems in the hippocampus (Ojika et al., 2000). PEBP is the precursor of HCNP. PEBP binds to PE in the membrane, and the autocatalytic release of HCNP is a consequence of this binding (Banfield et al., 1998). A decrease in the amount of PE may, therefore, reduce the level of HCNP during development and adversely affect cholinergic systems of the hippocampus, with a consequent outcome of the pathophysiology of autism. Thus, a possible role for PE, PEBP, and HCNP raises the possibility of an intricate system in the development of autism pathology. Because levels of PE may be controlled by diet, a possible diet-based strategy of treatment may be envisaged in this context.

CARBONIC ANHYDRASE

Carbonic anhydrase (CA) is a zinc-containing enzyme present in myelin (Sapirstein et al., 1978, 1984). Experiments with triethyltin, which induces edema of myelin, in knockout mice of CA have indicated a role for this enzyme in myelin compaction. In addition to the neurologic role, CA is present in various tissues, including pancreas and kidney tissues, that have a secretory function. In the pancreas, secretin enhances its activity. Thus, multiple phenotypic effects in autism may be related to the involvement of various isoforms of CA with tissue-specific effects. Thus, possible abnormalities in the constellation of CA isoforms present in the brain and other tissues need to be studied to understand their contribution to autism.

REELIN

Reelin is an extracellular signaling protein in the nervous system that affects CNS cytoarchitecture (Rice and Curran, 2001). Absence of this protein results in the

Reeler phenotype in mice. Altered levels of Reeler protein have been observed in postmortem brain tissues in autism (Fatemi, 2001). Although various context-dependent functions have been attributed to Reelin protein during development of the CNS (Rice and Curran, 2001), a direct effect of this protein in inducing the pathophysiology of autism remains to be identified. However, Reelin binds to beta integrins (Dulabon et al., 2000), which are involved in the initiation of myelin biosynthesis (Malek-Hedayat and Rome, 1994; Relvas et al., 2001; Tiwari-Woodruff et al., 2001). Thus, decreased amounts of Reelin in autism may not be sufficient for optimal signaling with integrins to regulate the ordered biosynthesis of myelin membrane. This possibility needs to be studied in detail.

CEREBROSIDES AND GM4

Because cerebrosides are precursors of GM4, a regulatory defect in this pathway may be partly responsible for alterations in myelin. The defect in this pathway would affect both the synthesis of cerebrosides and the ganglioside GM4. Both affect the integrity of myelin and its compaction.

Summary

Myelin is essential for fast neural transmission. The absence of functionally active myelin prevents effective and efficient transmission of impulses and activation of postsynaptic impulse-driven, stage-specific events needed during development of the CNS. Thus, alterations in myelin may make multifactorial contributions to the pathophysiology of autism and resultant behavioral phenotype.

Results from the analysis of autopsied corpus callosum, a tissue rich in myelin, from adult autistic individuals indicate selective alterations in glycolipids and phospholipids. The data suggest that myelin in autistic individuals is not mature and may not be fully functional, especially during the critical period of development. The results suggest clues for additional investigations in molecular neuropathology of white matter in autism.

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12

Positron Emission Tomography Studies of Autism

Diane C. Chugani, Ph.D.

Strategies for the Use of Positron Emission Tomography in Autism

Positron emission tomography (PET) has been applied to the study of autism using several strategies. The first strategy involves the search for global and regional differences in resting brain glucose metabolism or blood flow. A second strategy employs task performance during tracer uptake to investigate possible processing differences as indicated by changes in glucose metabolism or cerebral blood flow, focusing on high-functioning adult autistic subjects. The third strategy involves the definition of developmental changes in neurotransmitter/neurotrophic function with PET to determine whether there are differences with age in autistic children compared to more normally developing children. The first two strategies focus on detecting brain regions in which the mature autistic brain differs biochemically from normal in the resting or active state. The third strategy focuses on gaining an understanding of the process of altered postnatal brain development and possible regulators of that process. Finally, PET has been applied in several disorders in which there is a high incidence of autism.

PET is a method for measuring brain biochemistry in vivo through the use of biochemical substrates or ligands labeled with short-lived positron-emitting radioisotopes (Hoffman and Phelps, 1986; Langstrom and Dannals, 1995; Stocklin, 1995; Ter-Pogossian, 1995). The labeled biochemical of interest is injected intravenously, and the PET scanner is then used to image the distribution of the tracer in the body as the tracer is taken up in the brain, based on the localization of transporters, enzymes, receptors, or the like, specific for that tracer, which result in the concentration of the label in certain brain regions of interest. Although PET involves the exposure of subjects to a small dose of radioactivity and requires intravenous and, in some cases, arterial access, this method provides biochemical information in the living brain that cannot currently be obtained by any

other method. Ernst et al. (1998) reviewed studies of low-level radiation exposure with large sample sizes and long follow-up and concluded that health risks from low-level radiation at the level of exposure with PET scanning could not be detected above those of adverse events of daily life. In addition, they found no evidence that low levels of radiation were more harmful to children than to adults.

RESTING BRAIN GLUCOSE METABOLISM AND BLOOD FLOW

In the first study of glucose metabolism with the tracer 2-deoxy-2-[^{18}F]fluoro-D-glucose (FDG) in autism, Rumsey et al. (1985) reported diffusely increased glucose metabolism by approximately 20 percent in a group of 10 autistic men compared to 15 healthy gender- and age-matched control subjects. The finding of globally increased glucose metabolism has not been replicated in subsequent FDG PET studies reported (DeVolder et al., 1987; Herold et al., 1988; Siegel et al., 1992). However, there are methodologic differences in subsequent studies, and therefore, differences in global glucose metabolism in autistic adults cannot be discounted. For example, Herold et al. (1988) compared six male autistic subjects to six healthy males and 2 females. Similarly, Siegel et al. (1992) compared autistic adults (12 men, 4 women; age: 17–38 years) and normal controls (19 men, 7 women; mean age: 27 years) mixed for gender, and found no difference in global glucose metabolism. Because there are gender differences in glucose metabolism on the same order of magnitude of those Rumsey et al. (1985) reported between autistic and normal men (Baxter et al., 1987), the inclusion of females in control groups could mask a true global increase in glucose metabolism. DeVolder et al. (1987) reported no differences in global glucose metabolism in 18 autistic children (11 boys, 7 girls; age: 2–18 years) compared to a control group which was comprised of children (3 normal children aged 7, 14, and 15 years; 3 children with unilateral pathology aged 9, 12, and 12.5 years) with various brain pathologies, as well as 15 adults (mean age: 22 years). Few conclusions can be drawn from the DeVolder et al. study, because glucose metabolism shows marked changes with age (Chugani et al., 1987).

Horwitz et al. (1988) added four male autistic subjects to the series reported by Rumsey et al. (1985) and showed that the global brain glucose metabolic rate was 12 percent higher in the autistic group, a difference that was statistically significant. In addition, Horwitz et al. (1988) performed a correlation analysis that showed significantly fewer positive correlations between frontal and parietal cortices, with the most notable discrepancy found between the left and right inferior frontal regions. Furthermore, the thalamus and basal ganglia also showed less correlation with frontal and parietal cortices in the autistic group compared to the controls.

Focal abnormalities of glucose metabolism have been reported in a number of other studies in which global brain glucose metabolism was not addressed. Heh et al. (1989) studied glucose metabolism in the cerebellum based on neuropathologic data showing fewer Purkinje and granule cells in the cerebellum (Bauman and Kemper, 1985; Ritvo et al., 1986) and vermal cerebellar hypoplasia measured on magnetic resonance imaging (MRI) (Courchesne et al., 1988). However, Heh et al. (1989) showed no significant difference in mean glucose metabolic rates for cerebellar hemispheres or vermal lobes VI and VII in autistic subjects (5 men and 2 women; age: 19–36 years) compared to control subjects (7 men, 1 woman; age: 20–35 years). Schifter et al. (1994) studied a heterogeneous group of children (9 boys, 4 girls; age: 4–11 years) with autistic behavior coexisting with seizures, mental retardation, and neurologic abnormalities. Visual analysis of the FDG PET scans revealed that five of the 13 subjects had focal abnormalities located in different brain regions for each patient. Regions showing hypometabolism included the right cerebellum and left temporal/parietal/occipital cortices; right parietal cortex, bilateral thalamus, and left occipital cortex; right parietal and left temporal/parietal cortices; right parietal/occipital and left occipital cortices; and bilateral temporal lobes.

Zilbovicius et al. (2000) measured resting cerebral blood flow with [^{15}O]-water PET in autistic children with mental retardation (17 boys, 4 girls; age: 5–13 years; mean age: 8.4 years) and nonautistic children with idiopathic mental retardation (8 boys, 2 girls; age: 5–13 years; mean age: 8.1 years). The groups were compared using SPM 96, a voxel-based whole-brain analysis. Significantly lower blood flow in the right superior temporal sulcus (Brodmann's area 21), in the right superior temporal gyrus (Brodmann's area 44/22), and in the left superior temporal gyrus (Brodmann's area 44/22) was found in the autistic children. The analysis was repeated in an additional group of 12 autistic children (11 boys, 1 girl; age: 5–13 years; mean age: 7.4 years) and the same results were obtained. Each autistic child was also individually compared to the control group, and temporal lobe hypoperfusion was detected in 76 percent of the children. The temporal hypoperfusion was bilateral in nine children, but was found on the right side in only 16 children. Additional regions of low blood flow were detected in the individual analysis in the frontal, frontocerebellar, and occipital regions.

TASK PERFORMANCE DURING TRACER UPTAKE

Buchsbaum et al. (1992) applied a visual continuous performance task, which was associated with greater right than left hemisphere glucose metabolism in autistic subjects (5 men, 2 women; age: 19–36 years) than in their normal control

subjects (13 men; mean age: 24 years). Siegel et al. (1992) studied 16 high-functioning autistic adults (12 men, 4 women; age: 17–38 years) and 26 normal controls (19 men, 7 women; mean age: 27 years) and reported that autistic subjects had a left > right anterior rectal gyrus asymmetry, as opposed to the normal right > left asymmetry in that region. The autistic group also showed low glucose metabolism in the left posterior putamen and high glucose metabolism in the right posterior calcarine cortex. The same group (Siegel et al., 1995) studied glucose metabolism in 14 adults with a history of infantile autism (12 men, 3 women; age: 17–38 years; mean age: 24 years; 15 of 16 subjects previously reported by Siegel et al. [1992]) and reported that the autistic subjects showed abnormal thalamic glucose metabolism, and that correlations of task performance with pallidal metabolism suggested subcortical dysfunction during the attentional task in autism. Haznedar et al. (1997) performed MRI and glucose PET scans on seven high-functioning autistic patients (5 men, 2 women; mean age: 24.3 years) and seven sex- and age-matched normal adults. The right anterior cingulate was significantly smaller in relative volume and was metabolically less active in the autistic patients than in the normal subjects. However, these data were not corrected for partial volume effects (Hoffman et al., 1979), and the apparent decrease in glucose metabolism may be secondary to the reported volume decrease. More recently, Haznedar et al. (2000) expanded this study to include 10 subjects with high-functioning autism and seven with Asperger syndrome (15 men, 2 women; mean age: 27.7 years). The researchers reported significantly lower glucose metabolism in both the anterior and posterior cingulate gyri, whereas the reduced volume was found only in the right anterior cingulate gyrus. Thus, concerns regarding partial volume effects relate to only the right anterior cingulate.

In a functional mapping study using [^{15}O]-water PET, Happé et al. (1996) applied a "theory of mind" task that required attributing mental states to the characters of a narrative. The statistical parametric mapping analysis showed that the Asperger group (5 men; age: 20–27 years) showed a slightly different location of activation in inferior prefrontal cortex (Brodmann area 9 instead of 8) compared to the normal control group (6 men; age: 24–65 years).

Müller et al. (1999) studied auditory perception and receptive and expressive language in five high-functioning autistic adults (4 men, 1 woman; age: 18–31 years) compared to five normal men (age: 23–30 years) using a [^{15}O]-water activation paradigm. Scans were performed at rest, and while subjects listened to tones, listened to short sentences, repeated short sentences, and generated sentences. Analyses of peak activations revealed reduced or reversed dominance for language perception in the temporal cortex, and reduced activation of the auditory cortex and the cerebellum during acoustic stimulation in the autistic group.

Data from the four autistic men and five normal men were reanalyzed (Müller et al., 1998) to examine three predetermined regions of interest—the dentate nucleus of the cerebellum, the thalamus, and Brodmann area 46—based on serotonin synthesis studies showing abnormalities in these three regions in autistic boys (Chugani et al., 1997). The results of this study showed that the dorsolateral prefrontal cortex (area 46) and thalamus in the left hemisphere and the right dentate nucleus showed less activation in the autistic men than in the control group for sentence generation. In contrast, with sentence repetition, increases in blood flow were significantly larger in the left frontal cortex and right dentate nucleus in the autistic subjects than in the control group. These data suggest that the left frontal cortex, left thalamus, and right dentate nucleus showed atypical functional changes with language tasks in high-functioning autistic men.

Due to the small numbers of subjects, all of the functional mapping studies performed with PET should be considered pilot studies. However, this is a promising approach for high-functioning subjects who are able to cooperate with the performance demands of this type of study. Functional magnetic resonance imaging is now considered the method of choice for this type of study, because this method does not require the use of radioactive tracers.

DEFINITION OF DEVELOPMENTAL CHANGES IN NEUROTRANSMITTER OR NEUROTROPHIC FUNCTION WITH PET

Studies investigating alterations in neurotransmitters, hormones, and their metabolites in the blood, cerebrospinal fluid (CSF), and urine of autistic patients have been numerous and have provided some evidence for the potential involvement of several neurotransmitters in autism (for a review, see Anderson, 1994). Furthermore, given that there is evidence for dysfunction in widely distributed brain regions in autism (Minshew et al., 1997), the monoamine neurotransmitters are interesting candidates due to their widespread modulatory role in the brain. For this purpose, functional imaging has been used to examine the role for two monoamine transmitters, dopamine (Ernst et al., 1997) and serotonin (Chugani et al., 1997, 1999), in autism.

Ernst et al. (1997) studied 14 medication-free autistic children (8 boys, 6 girls; mean age: 13 years) and 10 healthy children (7 boys, 3 girls; mean age: 14 years) with [^{18}F]-labeled fluorodopa (F-DOPA) using PET. F-DOPA is a precursor of dopamine, which is taken up, metabolized, and stored by dopaminergic terminals. Ernst and colleagues calculated the ratios of F-DOPA activity (as the region of interest:occipital cortex ratio) measured between 90 and 120 minutes following tracer administration. Regions sampled were the caudate; putamen; midbrain; lateral and medial anterior prefrontal regions, regions rich in dopaminer-

gic terminals; and occipital cortex, a region poor in dopaminergic terminals. They reported a 39 percent reduction of the F-DOPA ratio in the anterior medial prefrontal cortex–occipital cortex in the autistic group. There were no significant differences in any of the other regions measured. These authors suggested that decreased dopaminergic function in prefrontal cortex may contribute to the cognitive impairment seen in autism.

Chugani et al. (1997, 1999) applied α[^{11}C]methyl-tryptophan ([^{11}C]AMT) as a PET tracer of serotonin synthesis (Diksic et al., 1990; Chugani and Muzik, 2000) in autistic children. Healthy, seizure-free children with autism (7 boys, 1 girl; age: 4–11 years) were compared to their healthy, nonautistic siblings (4 boys, 1 girl; age: 8–14 years). All of the autistic subjects and three of the five siblings were sedated with nembutal or midazolam. Significant asymmetries of [^{11}C]AMT standard uptake value (SUV) in the frontal cortex, thalamus, and cerebellum were measured in the seven autistic boys, but not in the one autistic girl studied, nor in four of the five siblings (Figure 12.1). Decreased [^{11}C]AMT accumulation was seen in the left frontal cortex and thalamus in five of seven autistic boys. This was accompanied by an elevated [^{11}C]AMT accumulation on the right in the cerebellum. The region of increased tracer accumulation in the cerebellum appeared to be in the dentate nucleus, based on coregistration with the MRI. In the remaining two autistic boys, [^{11}C]AMT accumulation was decreased in the *right* frontal cortex and thalamus and elevated in the left dentate nucleus. No asymmetries were seen in the frontal cortex or thalamus of the sibling group; however, one sibling showed an increased [^{11}C]AMT accumulation in the right dentate nucleus. Interestingly, this boy had a history of calendar calculation and

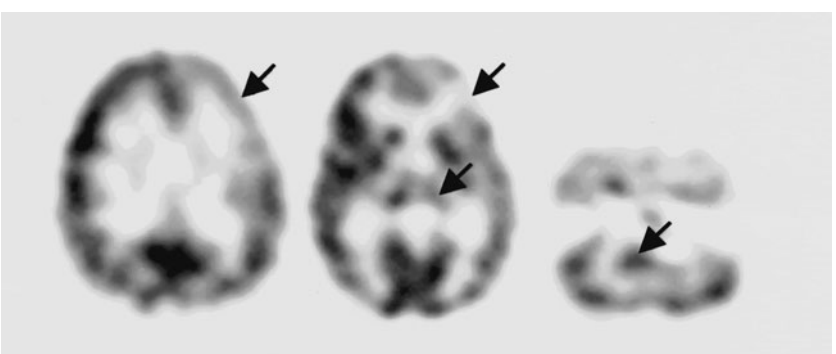

FIGURE 12.1. [^{11}C]AMT PET transaxial brain images from an autistic boy, shown at three levels. Arrows denote decreased [^{11}C]AMT accumulation in left frontal cortex and left thalamus and increased [^{11}C]AMT accumulation in right dentate nucleus in the autistic child (left side of image is the right side of the brain). *Source:* Chugani et al. (1997).

ritualistically lined up his toys, behaviors commonly seen in autistic children. The overall difference in asymmetry scores between the autistic boys and their siblings was found to be statistically significant, and regional asymmetry scores in the frontal cortex and thalamus were also found to differ significantly. The specificity of these abnormalities to serotonin synthesis was apparent when comparing the [^{11}C]AMT scans to the FDG PET and MRI scans, both of which were normal by visual examination, in the children studied.

Chugani et al. (1999) also measured whole-brain serotonin synthesis capacity in autistic and nonautistic children at different ages using [^{11}C]AMT and PET (Figure 12.2). Global brain values for serotonin synthesis capacity were obtained for 30 healthy, seizure-free autistic children (24 boys, 6 girls; age: 2–15 years), eight of their healthy, nonautistic siblings (6 boys, 2 girls; age: 2–14 years), and 16 epileptic children without autism (9 boys, 7 girl; age: 3 months–13 years). Children in the epilepsy group had medically intractable seizures, but patients with multiple anatomical brain lesions (e.g., tuberous sclerosis patients) were not included. Epileptic patients were taking at least one anticonvulsant medication. All of the autistic and epileptic subjects and four of the eight siblings were

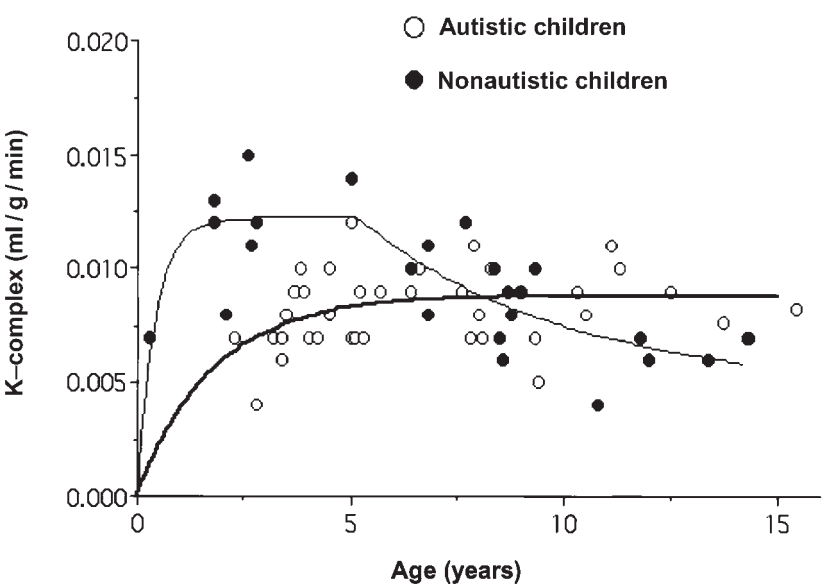

FIGURE 12.2. Serotonin synthesis capacity in children with autism ($N = 30$) and nonautistic children ($N = 24$; 8 siblings of children with autism and 16 children with epilepsy). Global brain values for serotonin synthesis capacity (K-complex, ml/g/min) were plotted as a function of age and fits were obtained for each group (heavy curve, autistic children; light curve, nonautistic controls).

sedated with nembutal or midazolam. For nonautistic children, serotonin synthesis capacity was more than 200 percent of adult values until the age of 5 years and then declined toward adult values. Serotonin synthesis capacity values declined at an earlier age in girls than in boys. In autistic children, serotonin synthesis capacity increased gradually between the ages of 2 years and 15 years to values 1.5 times the adult normal values and showed no gender difference. These data suggest that humans undergo a period of high brain serotonin synthesis capacity during childhood, and that this developmental process is disrupted in autistic children. Serotonin synthesis capacity was estimated in four high-functioning autistic adults (3 men, 1 woman; mean age: 26.5 years; mean full-scale IQ: 70) (D. C. Chugani et al., 1996). Values of serotonin synthesis capacity for different brain regions in one autistic woman studied fell within the range of values measured in normal adult females ($N = 5$). Comparisons made between autistic men and control men ($N = 5$) showed that mean serotonin synthesis capacity values were significantly higher for all brain regions in the autistic group.

Differences in serotonin synthesis with age are important in the light of the neurotropic role of serotonin during development. Indeed, manipulations of serotonin in animals during pre- and postnatal development can recapitulate some of the pathologic findings in autistic brain reported by Bauman and Kemper (1985, 1994). For example, Bauman and Kemper (1985) reported reduced neuronal cell size and increased cell number in the hippocampus of autistic brains. Treatment of pregnant rats with p-chlorophenylalanine to deplete serotonin resulted in a prolongation of the period of cell division in the pups in brain regions with dense serotonergic innervation, leading to increased neuronal cell numbers in the hippocampus, superior colliculus, and several thalamic nuclei (Lauder and Krebs, 1978). Bauman and Kemper (1994) also reported decreased complexity and extent of dendritic arbors in the hippocampus of autistic brains. In the animal literature, Yan et al. (1997) reported that depletion of serotonin with p-chlorophenylalanine or 5,7-dihydroxytryptamine in neonatal rat pups resulted in large decreases in the number of dendritic spines in the hippocampus. The focal decreases in [^{11}C]AMT that were observed in the cortex and thalamus might be interpreted in the light of developmental changes in serotonin mechanisms shown in animal studies. For example, it has been demonstrated recently that the serotonin transporter is transiently expressed by glutamatergic thalamocortical afferents (Bennett-Clarke et al., 1996; Lebrand et al., 1996) during the first 2 postnatal weeks in rats. During this period, these thalamocortical neurons take up and store serotonin, although they do not synthesize serotonin. Although the role of serotonin in glutamatergic neurons whose cell bodies are located in the sensory nuclei of the thalamus is not yet known, there is evidence that the serotonin concentration must be neither too high nor too low during

this period. Depletion of serotonin delays the development of the barrel fields of the rat somatosensory cortex (Blue et al., 1991; Osterheld-Haas and Hornung, 1996) and decreases the tangential arborization of the thalamocortical axons, resulting in reduced size of the barrels fields (Bennett-Clarke et al., 1994). Conversely, increased serotonin during this critical period increases tangential arborization of these axons, resulting in blurring the boundaries of the cortical barrels (Cases et al., 1995, 1996). Developmental changes in serotonergic mechanisms have also been reported in the cerebellum. 5HT1A receptors are transiently expressed in cerebellum during brain development. In rat pups, there is high expression of the 5HT1A receptor in the Purkinje cell layer between postnatal days 2 and 9, but there is no detectable 5HT1A receptor in the cerebellum of adults (Miquel et al., 1994). These data suggest an important role for serotonin in Purkinje cells of the cerebellum during brain development. Bauman and Kemper (1985) also reported decreased numbers of Purkinje cells in the cerebellum in autistic brains. These findings are particularly interesting in the light of the focal increase in [^{11}C]AMT demonstrated in the dentate nucleus of the cerebellum (Chugani et al., 1997), because Purkinje cells project to the dentate nucleus. In sum, the frontal cortex, thalamus, and cerebellum may be particularly sensitive to changes in serotonin synthesis during development, and the abnormalities in these regions measured with [^{11}C]AMT PET may stem from developmental alterations in serotonin synthesis.

PET in Disorders with a High Incidence of Autism

An association of autism in children with a history of infantile spasms has been long recognized (Riikonen and Amnell, 1981). H. T. Chugani et al. (1996) reported that 18 children (7 boys, 11 girls; age: 10 months–5 years) from a total of 110 children with a history of infantile spasms showed bilateral temporal lobe glucose hypometabolism on PET, with normal MRI scans. Long-term outcome data were obtained for 14 of the 18 children; 10 of the 14 children met DSM-IV criteria for autism. All 14 children had continued seizures and mental retardation. Two temporal lobe regions, the superior temporal gyrus and hippocampus, showed significant hypometabolism compared to age-matched controls. These observations are relevant not only because histologic studies of postmortem brain tissue from autistic subjects show abnormalities in the hippocampus (Bauman and Kemper, 1994), but also because recent studies using volumetric MRI in patients with fragile X syndrome have found abnormalities in the hippocampus (increased volume) and superior temporal gyrus (decreased volume) (Reiss et al., 1994).

Asano et al. (2001) examined the relationship between autistic behavior and epilepsy, structural brain abnormalities, and functional abnormalities of

glucose and tryptophan metabolism in *nonlesional* brain regions of interest in children with tuberous sclerosis complex (TSC). Children with TSC and intractable epilepsy underwent MRI and PET scans with FDG and α[^{11}C]methyl-L-tryptophan (AMT). Based on the results of Autism Diagnostic Interview—Revised, Gilliam Autism Rating Scale (GARS) and overall adaptive behavioral composite (OABC) from Vineland Adaptive Behavior Scale, subjects were divided into three groups: (1) autistic (OABC < 70; $N = 9$), (2) nonautistic/mentally retarded (OABC < 70; $N = 9$), and (3) normal intelligence (OABC ≥ 70; $N = 8$). MRI studies failed to demonstrate a significant association between the location of cortical brain lesions and autistic behavior, but there was a higher incidence of subependymal nodules adjacent to the caudate nucleus in the autistic group. PET studies showed that the autistic group had decreased FDG uptake in the lateral temporal gyri, increased FDG uptake in the deep cerebellar nuclei, and increased AMT uptake in the caudate nuclei, compared to the nonautistic mentally retarded group. In addition, a history of infantile spasms and decreased FDG uptake in the lateral temporal gyri were significantly associated with communication disturbance. Increased FDG uptake in the deep cerebellar nuclei and increased AMT uptake in the caudate nuclei were both significantly related to stereotyped behaviors and impaired social interaction, as well as communication disturbance. This study suggests that functional deficits in the lateral temporal gyri may be associated with communication delays and that functional imbalance in subcortical circuits may be associated with stereotyped behaviors and impaired social interaction in children with tuberous sclerosis complex.

Summary and Future Directions

The results of several of the studies of glucose metabolism and blood flow studies point to abnormalities in temporal cortical regions in both the resting state (Zilbovicius et al., 2000) and during activation (Müller et al., 1998). Temporal lobe hypometabolism was also found in autistic and mentally retarded children with TSC (Asano et al., 2001) and in a separate group of children with autism and infantile spasms (H. T. Chugani et al., 1996). These temporal lobe abnormalities are consistent with deficits in language perception and auditory processing in autistic subjects. Additional brain regions implicated by several tracers include the frontal cortex, thalamus, cerebellum, and cingulate. Abnormalities in several cortical-subcortical pathways, including the dentatothalamocortical pathway (Chugani et al., 1997), have been implicated in autism by PET studies. The finding of whole brain increases in glucose metabolism autistic adults (Rumsey et al., 1985) could be related to changes in the trajectory of the biochemical development of the brain in autism. The studies showing differences in changes of

serotonin synthesis with age are evidence of altered postnatal brain development (Chugani et al., 1999).

Thus, the application of functional neuroimaging with PET has shed new light on changes in brain function and biochemistry in the developing brain during maturation, as well as on deviation from normal developmental patterns in autistic children. The increasing sensitivity of the new generations of scanners allows a reduction in the radioactive dose administered, thus increasing the predilection to apply this imaging modality in children. In addition, the application of new image-processing techniques, such as voxel-based approaches, has increased the power to detect focal abnormalities.

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13

The Orbitofrontal-Amygdala System in Nonhuman Primates: Function, Development, and Early Insult

Jocelyne Bachevalier, Ph.D.

Autism is characterized by disruption in social-emotional behavior and communication. Across the spectrum of disability, autistic individuals have poor social and affective relatedness, difficulty developing and maintaining social relationships with peers, problems in the social use of language, unusual nonverbal behaviors (including gesture), and, in general, difficulty meeting cultural expectations for age-appropriate social behavior (for reviews, see Grossman et al., 1997; Loveland and Tunali-Kotoski, 1997). They have been shown to lack insight into the mental life of other people, to not appreciate others' points of view, and to have difficulty recognizing other people's emotions and reactions in social situations (Baron-Cohen et al., 1985; Hobson et al., 1988a, 1988b; Klin, 2000; Loveland et al., 2001). Behavioral self-regulation in response to the ever-changing conditions of the social world also appears to be severely affected in persons with autism (Loveland, 2001).

Given these disabilities, a neural model of brain dysfunction in autism must encompass not only those structures and systems that subserve social awareness and emotion recognition, but also those that subserve the regulation of behavior in response to a changing social environment (Schore, 1994, 1996; Bachevalier and Loveland, 2003). Furthermore, such a model must necessarily reflect that persons with autism change as they develop, not only because of experience, but also because brain maturation may be disrupted from very early in life and thus may yield to the formation of aberrant neural circuits that manifest themselves as enduring forms of psychopathology.

This chapter presents an overview of data from nonhuman primate research implicating the amygdala and the orbitofrontal cortex in the regulation of social-emotional cognition and behavior. It summarizes recent studies indicating that this neural system operates early in life and manifests critical periods of postnatal maturation, during which major refinements in the macaque behavioral repertoire coincide with refinements in the structural and functional develop-

ment of the amygdala and/or orbital frontal cortex. The final part of this chapter highlights a characterization of the behavioral deficits following early dysfunction of the orbitofrontal-amygdala circuit and of the widespread neural changes that follow this early dysfunction. This information may help to understand the neural systems that are dysfunctional in autism and in other developmental psychopathologies associated with drastic changes in social skills, such as Williams syndrome and schizophrenia (Machado and Bachevalier, 2003).

The Orbitofrontal-Amygdala System and Social Cognition in Primates

Broadly construed, social cognition rests on the ability to detect and interpret information about other individuals that is relevant to regulating one's own behavior according to the current emotional and social context. In the case of humans, the use of social information to self-regulate behavior is highly complex, reflecting not only emotional but also cognitive and cultural factors. Social cognition thus includes not only the ability to understand and reason about the cognitive mental states of other individuals but also the ability to identify emotional states, intentions, desires, and attitudes, and to use this information to guide behavior. Several recent reviews have suggested that social cognition is orchestrated by a complex neural network of interconnected structures, including cortical areas of the ventral surface of the frontal lobe (i.e., orbitofrontal cortex) and the amygdala within the temporal lobe, together with their interconnections with the cingulate cortex, hypothalamus, and brainstem (see Aggleton, 2000; Bachevalier, 2000; Bechara et al., 2000; Adolphs, 2001). Neurobehavioral, electrophysiologic, and neuroimaging studies (for a review, see Bachevalier and Loveland, 2003) indicate that the amygdala appears to detect the significance of objects or events for the individual, whereas the orbitofrontal cortex makes use of this information to guide and adjust behaviors appropriately in accordance with changing conditions (Schoenbaum et al., 1998; Bechara et al., 1999).

Etiologic studies have indicated that monkeys, like humans, live in social groups that are characterized by complex and dynamic social organization maintained through a variety of specific, long-term relationships between individual group members (DeWaal, 1989; Cheney and Seyfarth, 1990). To maintain these relationships, monkeys, like humans, need to perceive and use sensory cues from other individuals in the troop and adapt their responses to function in the social environment. Indeed, the presence of a stable social hierarchy in a group indicates that the individuals comprising the social group recognize one another and respond differentially, depending on with whom they are inter-

acting. Thus, the neural system mediating social skills in nonhuman primates is likely to be mediated by the same neural structures as those in humans.

The Emergence of Affective and Social Abilities in Nonhuman Primates

Beginning at birth, the nonhuman primate infant born into such a complex social group is faced with the developmental task of coming to respond differentially and appropriately to categories of social partners, as well as to individuals in those categories. Thus, during development, infants progressively learn complex rules that assure successful social relationships. All aspects of social cognition investigated thus far emphasized the remarkable similarities between infant macaques and human infants in this domain of cognition (for reviews, see Hinde and Spencer-Booth, 1967; Suomi, 1984).

From birth to 2 months of age, the newborn rhesus monkeys interact mostly with the mother, and their specific affective states are generally described as relaxed, alert, or upset. Only when they reached the second postnatal month did infant monkeys begin to expand their behavioral repertoires and exhibit more distinctive and diverse affective displays. Nevertheless, during the first two months, infant rhesus monkeys develop responsiveness to visual social cues from faces (Mendelson, 1982a, 1982b; Mendelson et al., 1982). They appear to perceive facial configurations and discriminate the direction of a conspecific's gaze by the first postnatal week, although they appreciate the social content of changes in gaze direction only by the third postnatal week. Concurrently, vocal recognition emerges (Masataka, 1986). The early development of facial and vocal recognition in infant macaques is in line with that observed in human infants (for reviews, see Nelson, 1985, 1993; Fernald, 1993). By the second postnatal month, infant primates begin to leave their mothers for brief exploratory excursions in their close surroundings, although, as do human infants, they still use their mothers as secure bases for their own exploration. Another affective trait that emerges during the second postnatal month is frustration, generally caused by the mother's attempting to restrain the infant from exploring the environment.

Fear of strangers emerges at 3 months and is expressed by the well-defined "fear grimace." This affective display characterizes a state of fear or wariness of social stimuli, which earlier in life elicited curious interest and sometimes exploration (Sackett, 1966, 1973). By this age, the infant monkeys can adaptatively modulate their defensive activity to meet changing environmental demands (Kalin and Shelton, 1989; Kalin, Shelton, and Takahashi, 1991).

Only by the age of 4 months does social play usually flourish. This affiliative activity will rapidly grow in both scope and frequency until adolescence, and constitutes the most prominent behavior pattern in a young monkey's interactions with its peers, siblings, and even some adults in its social group. Over this period of development, play behavior becomes more complex and results in the development of a greater degree of affective or emotional differentiation.

As plays develop, the infant-mother relationships change, with the mother beginning to reject more of the nursing attempts from her infant. To cope with this new situation, the infant seeks comfort from other individuals in the social troop or shows signs of aggressive responses toward peers or inanimate objects in their surroundings. Therefore, by 6–7 months, aggression becomes a clear-cut affective response in the monkey's behavioral repertoire, providing a better control of behavioral activity.

Given the relatively precocious development of some aspects of affective states (recognition of the social content of stimuli), one would expect some components of the orbitofrontal-amygdala system to be clearly functional by the end of the first month in monkeys. At the same time, other aspects of affective responses (e.g., fear responses) do not emerge before 3 months of age and yet others (e.g., aggressive responses), not before 6–7 months of age. This progressive development suggests that the neural circuit supporting social cognition matures in stages, as different components of the circuit become fully mature (for similar development in humans, see Schore, 1994, 1996). As summarized below, the experimental data to support this proposal are meager, but certainly offer a point of departure to initiate further investigations in this domain.

Maturation of the Obitofrontal-Amygdala System

In rhesus monkeys, neurons of the amygdala are generated very early in gestation (embryonic days 30–50 of the 165-day gestational period) and simultaneously within all amygdaloid nuclei (Kordower et al., 1992). At birth, all cytologic constituents of the amygdaloid nuclei are in place (Kling, 1966; for similar findings in humans, see Humphrey, 1968; Nikoliæ and Kostoviæ, 1986). In addition, opiate receptor as well as serotonergic distribution in the first 2 postnatal weeks is quite similar to that of adult monkeys (Bachevalier et al., 1986; Prather and Amaral, 2000). Despite this early ontogenetic maturation, the connectional system between the amygdala and temporal cortical areas continues to mature postnatally (Webster et al., 1991).

Although neurogenesis may occur simultaneously in all prefrontal areas (Mrzljak et al., 1990), different developmental sequences in the refinement of structural and neurochemical components could emerge between the orbito-

frontal and dorsolateral prefrontal cortices. Thus, connections between the dorsolateral prefrontal and orbitofrontal cortices become noticeable in the third trimester of gestation in monkeys and reach adult levels just weeks before birth (Schwartz and Goldman-Rakic, 1991), and corticocortical fibers become prominent and highly organized between the left and right orbitofrontal cortices (Goldman and Nauta, 1977). At birth, the temporal-orbital connections appear adultlike (Webster et al., 1994), although the feedback projections from the orbitofrontal cortex to temporal cortical regions continue to develop until the seventh week after birth (Rodman and Consuelos, 1994). The distribution of catecholamine innervation of the orbital frontal cortex is present in its adult form by 3 days after birth (Berger et al., 1990) even though changes in the laminar distribution of dopamine innervation can be seen until 6 weeks postnatally. Changes in neuropeptide distribution of the orbitofrontal cortex can be seen until adulthood (Hayashi and Oshima, 1986).

Such a progressive development of the orbitofrontal-amygdala system could, in fact, mediate the changes in social and emotional behavior over the first several years of a macaque's life. Thus, the emergence of fear and defensive responses around the third month corresponds to the age at which rhesus mothers generally allow their infants to venture off with their peers and is in close association with the emergence of fear of strangers. This affective development indicates that, by this age, infant monkeys respond with different emotional displays to specific facial expressions from other conspecifics, suggesting that the innate wiring and learned skills needed to discriminate threatening cues are in place. It also suggests that some important changes in the neural organization of the network assuring defensive responses have occurred. It is likely that these changes encompass a refinement of connectivity not only within the amygdala but also between the amygdala and other areas of the brain. Given the participation of the prefrontal cortex in the interpretation of sensory stimuli and in the inhibition of maladaptive responses, important changes could occur between the amygdala and orbitofrontal cortex interactions around this postnatal age. Very little is known in this respect, except that around this age, the ability to inhibit inappropriate responses (a cognitive skill dependent on the integrity of the orbital prefrontal cortex) emerges in monkeys (Goldman, 1971). Thus, the maturation of the orbital prefrontal cortex and of its connections with the amygdala around 3 months of age would permit the animal to modulate affective responses according to constant changes in social cues provided by others and to contend successfully with danger. In sum, there exist critical periods in the development of emotional responses and social skills in nonhuman primates that appear to parallel critical periods in the maturation of the orbitofrontal-amygdala system.

Early Dysfunction of the Orbitofrontal-Amygdala System

Kling and collaborators (Kling and Green, 1967; Kling, 1972) followed the development of four monkeys amygdalectomized during infancy over a period of 2 years and noted that, when returned to their mothers, the infant monkeys could successfully be raised maternally. They displayed normal nipple orientation, sucking, and grasping, with a somatic and affective development grossly in the normal range. In addition, following repeated presentations of inedible objects, these operated animals did not display the typical compulsive oral behavior seen in amygdalectomized adult monkeys.

More systematic and detailed investigation of the effects of neonatal amygdala lesions in rhesus monkeys (for a review, see Thompson, 1981) clearly showed that bilateral amygdalectomy does not leave the subject unharmed, even when the surgery is performed during infancy. Thus, on observation of six females that had sustained bilateral aspiration lesions of the amygdaloid complex during the third postnatal month, significant alterations in social affiliations were found, and these changes became increasingly more evident with age. In the first few months after surgery, the infant monkeys displayed more fear responses during social encounters than did control monkeys with whom they were paired, and the fear responses made by the operated monkeys toward controls increased whenever the control animals became more active, even though this activity was not overly aggressive. These enhanced fear reactions first appeared 3–5 months after surgery and intensified dramatically thereafter. Responses to novel stimuli in the absence of other monkeys revealed the opposite picture, however: operated monkeys made fewer fear responses than did controls and showed signs of hyperactivity. These findings indicate that the early amygdala lesions did not influence the emergence of fear responses around the appropriate age at which they normally appeared. Nevertheless, the early lesions did affect the magnitude of these responses in the presence of a peer, suggesting that the operated animals may have difficulty in evaluating the social cues expressed by their normal peers. These results were replicated using more selective damage to the amygdala at approximately 2 weeks of age (Prather et al., 2001). Thus, early amygdala lesions do not abolish the normal emergence of fear responses at about 3 months of age, but do affect the *magnitude* of the fear responses displayed in the presence of peers and novel objects. This suggests that the operated animals have difficulty in evaluating potentially threatening situations and/or implementing behaviors that will likely keep them from harm.

When retested in adulthood, the monkeys operated on in infancy showed transient hyperactivity (Thompson, 1981). They were also subordinate to normal controls, but expressed less fear than did the controls when briefly placed with

an unfamiliar aggressive animal. This increase in subordinate responses in the operated animals suggests that the amygdala lesions may have affected the normal development of aggressive responses, resulting in subordination and low social status in the operated animals. Finally, the behavioral abnormalities in monkeys with early amygdala lesions did not differ from those of monkeys that had sustained the same lesions in adulthood. Thus, overall, these data suggest that the amygdala may be operating early in life to regulate affective responses and to establish and maintain social status.

These changes in emotionality and sociality seen after early damage to the amygdala were substantiated and extended by our own studies on the development of social interactions in infant monkeys that were amygdalectomized in the first postnatal month (Bachevalier, 1994). At 2 months of age, amygdalectomized monkeys were more inactive than controls, but had normal amounts of total social contacts, although the social interactions were almost exclusively initiated by the controls. However, at 6 months of age, the changes in general activity disappeared, but social interactions were dramatically reduced and were accompanied by a significant increase of dominant approaches from the controls and a mild increase in active withdrawals from social contacts by the operated animals. Presumably, as a result of a marked reduction in affiliative behaviors, both operated and control animals displayed an increased amount of stereotyped behaviors, more so in the operated animals than in the controls. The early amygdala damage also dramatically altered vocal responses to social separations (Newman and Bachevalier, 1997), a finding consistent with the more general deficiency in social affiliation found in these early-operated animals.

At adulthood, the most striking change was the almost complete lack of social interactions between operated and control monkeys. Presumably, by this age, the normal animals had learned to cope with the abnormal affective and social responses of the operated animals and did not interact any more with them. Thus, as shown earlier by Thompson (1981), early damage to the amygdala results in increasingly more profound changes in affective responses and social behavior as the animals matured.

When neonatal lesions were extended to include the ventromedial temporal cortical areas and hippocampus, the behavioral, emotional, and social changes were even more severe, including a lack of social skills, flat affect, and significant increase in locomotor stereotypies and self-directed activities (Bachevalier, 1991, 1994; Bachevalier et al., 2001). By contrast, as shown by Thompson (1981) and Prather and colleagues (2001), these early lesions did not yield the lack of fear responses and hyperorality commonly seen after late medial temporal lobe lesions in monkeys (Meunier et al., 2003). Finally, when the animals with neonatal medial temporal lobe lesions were retested in social situations as they

reached adulthood, the loss of social interactions was substantially more severe in magnitude in adults with early lesions than in adults with similar lesions acquired in adulthood, and the stereotypies were evident after early lesions but not after late lesions (Málková et al., 1997).

The effects of early damage to the orbitofrontal cortex on social and emotional behavior have not been extensively studied. Dyadic social interactions between monkeys with orbitofrontal cortex damage induced at 2 months of age and unoperated controls were examined at approximately 8 months of age (Bowden et al., 1971). The operated monkeys showed higher frequencies of huddling alone and initiated fewer behaviors overall than did controls during social encounters. In contrast to this pattern of hypoactivity in social situations, when the operated monkeys were observed while alone, they showed hyperactivity (mostly elevated levels of locomotion) relative to controls. These data suggest that, unlike early damage to the amygdala, which appears to dysregulate fear *and* social behavior, early damage to the orbitofrontal cortex impairs only the initiation of social interactions. Furthermore, although the long-term effect of neonatal orbitofrontal cortex damage on social and emotional behaviors is still unknown, tests of behavioral inhibition and extinction indicate that, unlike the situation with amygdala damage discussed earlier, orbitofrontal cortex lesions produced after 8 months of age result in greater impairment than when the same lesions are inflicted just after birth (Jones and Mishkin, 1972; Goldman et al., 1974). The message emerging from the effects of early damage to the orbital frontal cortex is that if a lesion affects a brain structure or region that has yet to mature functionally, the effects of the lesion may remain silent until that structure or system matures. Thus, the pattern of results suggests that although the macaque amygdala appears to be functionally mature just after birth, the macaque orbitofrontal cortex may not achieve functional maturity until approximately 8–12 months after birth.

Widespread Impact of Early Insult to the Orbitofrontal-Amygdala System

Several behavioral lesion experiments have shown that longitudinal follow-up of animals with early brain lesions enables investigators to chart and observe the changing expression of the early lesions as development itself modifies behavior in general. Thus, behavioral lesion experiments provide an opportunity to discover how brain and behavior reorganize following insults at different points during development. Although the effects of neonatal damage to the amygdala and orbitofrontal cortex on the mechanisms of brain reorganization have yet to

be determined, there exists mounting evidence that early damage to medial temporal lobe structures, including the amygdala, has widespread repercussions on other neural systems. Thus, insults to the medial temporal lobe in infancy yielded a delayed maturation of the dorsolateral prefrontal cortex (Bertolino et al., 1997), characterized by changes in dopamine innervation (Chlan-Fourney et al., 2000) and associated with a dysregulation of striatal dopaminergic neurotransmission (Saunders et al., 1998; Heintz et al., 1999). Such dysregulation of prefrontal-striatal dopamine transmission suggests that the lack of functional inputs from the medial temporal structures prevents the prefrontal cortex from undergoing proper neuronal development. More broadly, the data imply that a fixed dysfunction localized to one of the nodes of a neural circuit can influence other areas of the circuit, especially if this dysfunction occurs early in development. Although much information needs to be gathered in this area of research, unraveling the reorganization in brain structures after lesions inflicted at different time points in development should greatly contribute to understanding the ontogenesis of developmental psychopathologies, such as autism.

Conclusion

Recent clinical and experimental studies lend support to the theory that medial temporal lobe structures and the orbitofrontal region are involved in the genesis of autism (for reviews, see Dawson, 1996; Baron-Cohen et al., 1999, 2000; Bachevalier and Loveland, 2003). Nevertheless, the biological basis for the behavioral syndrome of autism has been difficult to identify, in part because in a developmental disorder, brain differences are likely to be subtle, complex, and widely distributed rather than being localized. Thus, animal models of disordered brain development may have much to contribute to the study of such disorders as autism, because they provide the opportunity to observe directly the relationships of brain and behavior to development. Of particular importance are (Cicchetti and Tucker, 1994; Cicchetti and Cannon, 1999; Sánchez et al., 2001):

- The detailed characterization of major developmental stages in the neural structures implicated in social cognition, and the identification of critical developmental periods during which the self-reorganizing brain may allow for greater functional plasticity or more effective interventions;
- The description of behavioral abnormalities caused by damaging the amygdala, orbitofrontal cortex, or both, before and after critical developmental periods;
- The identification of the ability (or inability) of the brain to spontaneously reorganize to compensate for early impairment;

- The assessment of brain reorganization as a result of lesions to early-developing structures;
- The demonstration of how environmental factors may affect normal brain development and its functions across the life span; and
- The study of how self-regulating functions of brain development may be influenced by environmental factors at specific periods of development.

These issues are of special interest in the study of autism, because they reflect on some of the most difficult developmental questions surrounding this spectrum of disorder: Why are specific social-emotional deficits present in autism and so difficult to remedy? How can we account for the developmental variations among people with autism, such as the extent to which intellectual deficits are present?

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14

An Animal Model of Virus-Induced Autism: Borna Disease Virus Infection of the Neonatal Rat

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A number of neurodevelopmental disorders, including autism, have been associated with early brain damage following exposure to unfavorable environmental factors (Trottier et al., 1999; Lord et al., 2000). Because the pathogenesis of autistic spectrum disorder (ASD) appears to represent a continuum of unfolding events in which primary and secondary mechanisms are intimately interwoven, it is extremely difficult to draw any meaningful conclusions about the causative relationship between the often remote etiology (i.e., early brain injury) and the end-point disease phenotypes (Yeung-Courchesne and Courchesne, 1997). Thus, pathogenic treatments of early injury are lacking and have to be limited to the amelioration of secondary alterations expressed as signs and symptoms of disease (Baren, 1999; Tsai, 1999).

Although animal models cannot duplicate a human psychiatric disease, appropriate animal models can be used as "simulations" for studying the pathogenesis of behavioral diseases and can provide clues to develop pathogenically relevant novel therapeutic or preventative interventions (Ellenbroek and Cools, 1995; Geyer and Markou, 1995). Modeling human behavioral disorders is based on various experimental approaches that have both unique benefits and drawbacks. For example, animal models that predict pharmacologic outcomes as seen in humans are considered to have the predictive validity (Matthysse, 1986). Although these models are useful for screening novel pharmacologic treatments, they usually give few insights into the pathogenesis of the disease in question. Animal models demonstrating symptom similarity to a disease are characterized by face validity (i.e., behavioral isomorphism). Although behavioral isomorphism is an important phenomenon for studying the underlying mechanisms, achieving symptom or syndrome similarities can be both difficult and deceptive (Ellenbroek and Cools, 1995). On the one hand, some cognitive deficits can be hardly modeled in animals (e.g., language abnormalities). On the other hand, although such abnormalities as hyperactivity are readily induced by

various manipulations, studying the *mechanisms* of hyperactivity may or may not lead to revealing the pathogenic events specific to a disease of interest (e.g., autism). Thus, animal models that reproduce key pathogenic processes underlying a disease (construct validity) are thought to represent the most insightful group of animal models (Elsner, 1992; Ellenbroek and Cools, 1995). Because autism is believed to be a developmental disorder associated with early brain damage, studies of humans diagnosed with autism are typically performed "post hoc"; in contrast, animal models of abnormal brain and behavior development are likely to provide a better understanding of the pathophysiology of ASD (Yeung-Courchesne and Courchesne, 1997; Trottier et al., 1999).

The etiology of autism-like neurodevelopmental damage is likely to be heterogeneous (e.g., genetic defects, drugs, viruses), suggesting that even disparate etiologic agents are acting through similar, if not identical, pathogenic pathways (Ciaranello and Ciaranello, 1995; Trottier et al., 1999). However, enough differences exist among the various forms of ASD that modeling autism-like brain and behavioral abnormalities is likely to need multiple approaches to fully mimic and evaluate all the pathogenic processes involved (Rapin and Katzman, 1998; Lord et al., 2000). Current animal model systems of developmental disorders have used various gene-knockout approaches (Lijam et al., 1997; Young, 2001), as well as numerous physical and chemical agents (Altman, 1987; Binkerd et al., 1988; Miller and Stromland, 1993; Bachevalier, 1994) to derail normal brain development and produce various behavioral deficits. Interestingly, despite the ability of wild-type viruses (e.g., rubella, herpes simplex, measles) to induce central nervous system (CNS) injury with autistic disease phenotype, thus providing the biologic plausibility for using virus infection-based animal models (Chess, 1977; Mohammed et al., 1993; Yolken and Torrey, 1995; Johnson, 1998; Gillberg, 1999), there are few animal models of developmental disorders using viruses as teratogens (Crnic and Pfizer, 1988; Fatemi et al., 2000; Pearce, 2001). Over the past 10 years, characterization of developmental damage in Borna disease virus (BDV)-infected newborn rats has provided a model for exploring the pathogenesis of ASD.

Borna Disease Virus Infection

This chapter critically reviews neuroanatomic, behavioral, neurochemical, and immunologic features of the animal model of ASD based on developmental brain injury following noninflammatory, persistent CNS infection of neonatal rats with an experimental teratogen: a 8.9-kb, nonsegmented, negative-strand, round, enveloped RNA virus, BDV (Carbone et al., 1991; de la Torre, 1994). Neonatal BDV infection does not cause death or generalized brain damage and is not

associated with leucocyte infiltration in the brain parenchyma (Hirano et al., 1983; Narayan et al., 1983; Carbone et al., 1991). Instead, neonatal BDV infection produces regional brain damage and distinct behavioral disorders in rats, allowing study of the brain-behavioral relationship from a developmental perspective by using a more naturalistic approach for modeling brain injury, compared with lesion or pharmacologic manipulations (Pletnikov et al., 2002a).

Autistic-like Features of Neonatal Borna Disease Virus Infection

APPEARANCE AND PHYSICAL GROWTH

Intracranial inoculation of newborn rats with BDV leads to lifelong infection of the brain without classical such signs of CNS infection as malaise, fever, or anorexia (Hirano et al., 1983; Narayan et al., 1983; Carbone et al., 1991). Neonatally BDV-infected rats have normal body shape and proportion but are smaller than control animals, as evidenced by the difference in the overall body size and weight observed as early as day 4 postinfection (Bautista et al., 1995; Hornig et al., 1999; Pletnikov et al., 2001). Because there are no virus-associated differences in the amount of food ingested or levels of glucose, growth hormone, and insulin-like growth factor-1 in BDV-infected and control rats, the reasons for runting remain unclear (Bautista et al., 1995). Significantly, our preliminary experiments showed that, although malnutrition can lead to growth inhibition and brain developmental abnormalities, BDV-associated inhibition of postnatal weight gain alone does not explain the selective autistic-like alterations in rats (Dietz and Pletnikov, 2003).

NEUROPATHOLOGY

Available histopathologic and imaging data have shown that the hippocampus, cerebellum, amygdala, frontal lobes, basal ganglia, and brainstem are the most consistently affected brain regions in autistic patients (Bauman et al., 1997; Courchesne, 1997; Rapin and Katzman, 1998; Lord et al., 2000). Similarly, virus-associated abnormalities in the cerebellum, hippocampus, and neocortex have been described in the BDV model.

Cerebellum. Cerebellar alterations, including Purkinje cell loss, have frequently been found in autism (Bauman et al., 1997; Courchesne, 1997). In the BDV model, cerebellar damage follows a specific time course after intracranial inoculation with the virus (Carbone et al., 1991; Bautista et al., 1995; Eisenmann et al., 1999; Hornig et al., 1999). After a brief period of seemingly normal development for the first 2 postnatal weeks, BDV-infected cerebellum starts to show evidence

of arrested development (e.g., stunted size, decreased foliation, thinned and irregular layers in the folia). By the end of the third postnatal week (i.e., the completion of the cerebellum development), BDV-infected cerebellum is characterized by a size reduction in the molecular layer and internal granule cell layer (Bautista et al., 1995). Infection of Purkinje cells appears to lead to a gradual elimination of the majority of these cells by 4–7 months postinfection (Eisenman et al., 1999; Zocher et al., 2000; Pletnikov et al., 2002b) (Figure 14.1). Interestingly, spared Purkinje cells are somewhat variably distributed in the cerebellum, with some lobules containing a fairly uniform complement of Purkinje cells, whereas other lobules are severely deficient in Purkinje cell (Eisenmann et al., 1999; Zocher et al., 2000). Although the granule cells in the external germinal layer and internal granule cell layer do not seem to contain BDV antigens in vivo or in vitro (Bautista et al., 1995; Weissenbock et al., 2000), there seems to be a drop-out of granule cells as well (Zocher et al., 2000); however, quantitative examinations are necessary to ascertain this notion. The mechanisms of neuronal loss in the cerebellum are unclear. Although one report has pointed to apoptosis as a leading process of cell death (Hornig et al., 1999), another study failed to demonstrate apoptotic elimination of Purkinje cells (Zocher et al., 2000).

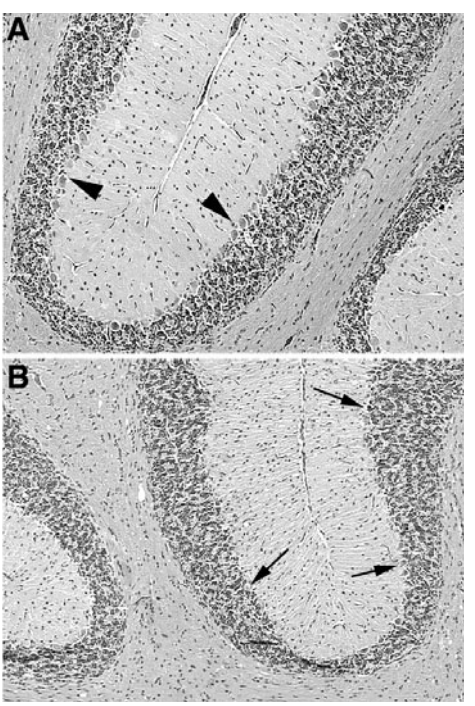

FIGURE 14.1. BDV-induced loss of Purkinje cells at 120 days postinfection. Representative images of cerebellar sections from a sham-inoculated (A) and a BDV-infected (B) rat. Note the intact layer of Purkinje cells in the sham-inoculated rat (arrowheads) and rare Purkinje cells in the BDV-infected rat (arrows).

Hippocampus. Neuropathologic studies of the limbic system in autism have found decreased neuronal size, increased neuronal packing density, and decreased complexity of dendritic arbors in the hippocampus, amygdala, and other limbic structures (Bauman et al., 1997). These findings are suggestive of a developmental curtailment in the maturation of the neurons and neuropil (Lord et al., 2000). Magnetic resonance imaging findings largely corroborated the neuropathologic data, revealing a reduction in the volumes of various areas of the limbic system (Haznedar et al., 2000; Saitoh et al., 2001; Sparks et al., 2002). Neonatal BDV infection is associated with a continuing degeneration of granule cells of the hippocampal dentate gyrus (DG), reaching an 80 percent neuronal loss by day 120 postinfection (Pletnikov et al., 2002a). Disappearing DG neurons are replaced by glial cells, including astrocytes and microglia (Carbone et al., 1991; Weissenbock et al., 2000). Qualitative evaluation of neurons in the CA1–4 regions of the hippocampus showed less dramatic, but clear, neuronal deterioration as well (Carbone, unpublished data). Apoptosis has been implicated in the degeneration of DG in BDV-infected rats (Carbone et al., 1991; Weisenbock et al., 2000). In addition, a significant decrease in immunostaining for synaptic markers, growth-associated protein (GAP-43), and synaptophysin has been reported, indicating synaptic pathology in the hippocampus (Gonzalez-Dunia et al., 2000). Whether synaptic alterations result from neuronal death or are due to direct virus toxicity remains to be seen.

Cortex. Despite the mixed reports on the nature of cortical abnormalities in autism, certain cognitive and behavioral deficits clearly suggest frontal and temporal cortex abnormalities (Shalom, 1999; Casanova et al., 2002; Skoyles, 2002). In contrast to the cerebellum and hippocampus, BDV-associated maldevelopment of the cortex has been relatively overlooked. What has been reported is that there is cortical shrinkage of 30 percent and a selective loss of cells with diameters greater than 100 μm in adult rats following neonatal infection with BDV (Gonzalez-Dunia et al., 2000; Pletnikov et al., 2002b). Both apoptosis of pyramidal neurons and alterations in neuropil might be responsible for cortical shrinkage (Weissenbock et al., 2000).

Summary. Neonatal BDV infection causes selective developmental damage to the cortex, hippocampus, and cerebellum (i.e., the brain regions that continue to develop after birth and may be sensitive to environmental insults) (Altman and Bayer, 1997). Although the available data do not seem to indicate significant BDV-associated cell loss in other regions of the brain, quantitative studies would be necessary to evaluate putative cell loss elsewhere. Both direct viral toxicity due to virus replication in neurons and indirect effects of soluble factors secreted by resident immune cells (e.g., cytokines, reactive oxygen intermediates) could be responsible for neuronal death in BDV-infected rats (see below).

BEHAVIORAL DEFICITS

BDV-induced neuroanatomic damage may underlie behavioral abnormalities observed in rats. Many of the behavioral deficits are reminiscent of several core and associated features of autism and include deficient social behaviors (Pletnikov et al., 1999a), disturbed emotionality (Pletnikov et al., 1999b), cognitive deficits (Dittrich et al., 1989; Pletnikov et al., 1999b; Rubin et al., 1999), and locomotor hyperactivity and stereotypy (Bautista et al., 1994; Hornig et al., 1999; Pletnikov et al., 2002b).

Abnormal Social Interaction. Deficient social interaction is a major behavioral abnormality in autism (Rapin and Katzman, 1998; Lord et al., 2000). Autistic children show a lack of appropriate response to social stimuli, fail to achieve joint attention (wherein other individuals are "drawn into" paying attention to the same object), fail to imitate the parental actions, and have abnormal expression of social attachment (Ciaranello and Ciaranello, 1995; Lord et al., 2000). Play behavior is particularly abnormal in autism and is characterized by a lack of social engagement, and, instead, a tendency toward repetitive, stereotyped, and nonfunctional object manipulation (Rapin and Katzman, 1998).

Thus, deficits in social behaviors are necessary to support the face validity of any animal model of autism. Neonatal BDV infection induced abnormal social interaction and communication in rats when tested at day 30–35 postinfection using the resident/intruder paradigm (Pletnikov et al., 1999a). A resident rat was isolated for 1 week to increase social motivation (Pankseep et al., 1984). An unfamiliar rat (intruder) was then placed in the resident's cage. This situation stimulates social interactions between rats, resulting in play behavior in young rats (measured as number of "pins," similar to a pin observed in a wrestling match). When play activity of the resident was analyzed, the control rat pairs exhibited significantly more pins than the pairs in which one or both of the rats was infected with BDV. One of the reasons for reduced play activity in the BDV-infected rats could be a decreased drive to engage their partner in social interaction. To measure soliciting activity in rats, such play-soliciting responses as pounce, crawl over/under, and darting were counted. This approach has revealed more play-soliciting responses in the control resident rats compared to the BDV-infected resident rats, indicating play solicitation was offered by the control rat and that reduction in play behavior was due to a decreased readiness to engage in play on the part of the BDV-infected rats. Significantly, the reduced play activity in BDV-infected rats was not associated with diminished locomotion or "nonplay" social behavior. In fact, compared to control animals, nonplay social interaction was elevated in BDV-infected rats, suggesting that the entire organization of the timely expression of differ-

ent types of social interaction was significantly affected by BDV infection (Pletnikov et al., 1999a).

Disturbed Emotionality. Hypersensitivity to changes in the environment, and the ensuing anxiety and fear, is one of the hallmarks of the emotional disturbances in autism (Rapin and Katzman, 1998; Lord et al., 2000). Patients with autism show a wide range of emotional deficits, including inability to tolerate changes or transitions, reacting to imminent or recent changes with strong anxiety states (Rapin and Katzman, 1998; Lord et al., 2000). Similar to autistic patients, neonatally BDV-infected rats exhibit a number of responses that suggest disturbed emotionality and hyperreactivity to aversive and/or novel stimulation. Damage to the hippocampus and cerebellum, as well as abnormal functioning of the amygdala, due to virus infection have been put forward to explain emotional abnormalities in BDV-infected rats (Pletnikov et al., 1999b).

Cognitive Deficits. Early damage to the limbic system might disturb acquisition and/or processing of novel information from daily life experiences, leading to abnormal memory functioning in autistic individuals (Lord et al., 2000; Minshew and Goldstein, 2001). Deficits in the cognitive domain have been demonstrated in neonatally BDV-infected rats in a number of behavioral tasks requiring the proper functioning of the corticolimbic system. For example, neonatal BDV infection produces deficits in spatial/discrimination learning and memory in the Y-maze, the hole board test (Dittrich et al., 1989), and in the Morris water maze (Rubin et al., 1999). Abnormal contextual learning and memory have also been demonstrated in BDV-infected rats by using the fear conditioning paradigm. Specifically, BDV-infected rats exhibited decreased species-specific fear responses to the test chamber previously paired with aversive stimuli (e.g., sudden loud noise), indicating deficits in contextual fear conditioning (e.g., BDV-infected rats failed to respond to the fearful environment with complete immobility) (Pletnikov et al., 1999b). Notably, an autonomic component (i.e., defecation) of contextual conditioned fear was spared, suggesting selectivity in virus-induced alterations in the brain system mediating fear conditioning (Pletnikov et al., 1999b).

Movement Disorders. Despite the heterogeneity of clinical manifestations of autism, some autistic patients exhibit abnormal movements that may be unique for the disorder. In young children, common findings include increased joint laxity and hypotonia, clumsiness, apraxia, and toe walking (Brasic, 1999; Rapin and Katzman, 1998). A variety of stereotypic movements, such as hand flapping, pacing, spinning, running in circles, and self-injuring behaviors, may be pathognomic of autism (Brasic, 1999; Pierce and Courchesne, 2001). Although the neurologic bases of movement anomalies in autism remain unclear, the cerebellar pathology has been implicated in several reports (Courchesne, 1997; Pierce and

Courchesne, 2001; Skoyles, 2002). In the rat, cerebellum-controlled behaviors have a discrete, organized pattern of evolution, paralleling the physical development of the cerebellum (Altman and Bayer, 1997). Neonatally BDV-infected rats show no evidence of gross ataxia but exhibit behavioral deficits that could be linked to developmental cerebellar injury (Bautista et al., 1995; Hornig et al., 1999; Pletnikov et al., 2001). A recent study has revealed a time course of sensorimotor deficits in developing BDV-infected rats by evaluating proprioception, motor strength, and coordination skills (Pletnikov et al., 2001). BDV-induced motor impairments were selective and correlated with the time course of BDV damage to the cerebellum. BDV-induced motor deficits were not seen until the end of the second postnatal week. By the third postnatal week, BDV-infected rats displayed abnormalities in negative geotropism and fore and hind limb placing and grasping. BDV-infected rats were also deficient in the ability to hold on to a bar and to cross a suspended bar, indicating hind limb weakness and lack of hind limb coordination that have been reported to be the most pronounced deficits of early cerebellar injury (Caston et al., 1998).

Thus, an array of BDV-induced behavioral deficits in developing and adult rats is reminiscent of core and associated features of autism, indicating the face validity of the model. Importantly, behavioral isomorphism of the BDV model is associated with BDV-associated autistic-like damage, indicating a combination of face and construct validity for this animal model.

NEUROCHEMICAL ALTERATIONS

Despite more than 30 years of research on the neurochemistry of autism, only a few highly reproducible neurochemical abnormalities have been observed, largely from indirect (blood or cerebrospinal fluid) measurements or observations of drug effects; for example, monoamine (dopamine, norepinephrine [NE], serotonin) and neuropeptide (opioids) alterations (Anderson, 1994; Chugani et al., 1997). BDV-induced selective regional alterations in the serotonin (5-HT) and NE neurotransmission in developing and adult BDV-infected rats, as evidenced by elevated tissue contents of 5-HT and NE in synaptic terminals and in the areas of cell bodies, along with alterations in the density of pre- and postsynaptic 5-HT receptors, have been documented (Pletnikov et al., 2000, 2002c) (Figure 14.2). These findings appear to suggest that neonatal BDV infection produces a decrease in 5-HT neurotransmission due to two major pathogenic events: neuronal loss in brain regions damaged by the virus and decreased release and/or deficits in axonal transportation of 5-HT because of the infection of 5-HT neurons in the raphe nuclei (Pletnikov et al., 2002c). Although it is difficult to compare these data with the limited and indirect data on 5-HT alterations in autistic children,

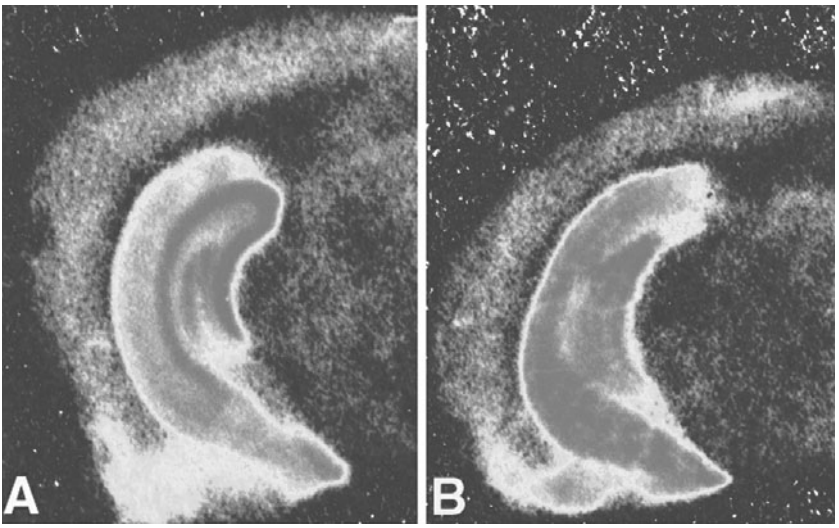

FIGURE 14.2. BDV-induced up-regulation of postsynaptic 5-HT1a receptors in the limbic system at 30 days postinfection. Representative autoradiograms of specific binding of [^{3}H]-8-OH-DPAT to postsynaptic 5-HT1a receptors in the limbic system in a sham-inoculated (A) and a BDV-infected (B) rat. Note the BDV-induced increase in [^{3}H]-8-OH-DPAT binding in different areas of the hippocampus.

specific BDV-associated serotonin disturbances, if confirmed in future human studies, may provide insight into what role the 5-HT system might play in mediating a developmental injury and resulting behavioral abnormalities.

GENETIC BACKGROUND–ENVIRONMENT INTERACTION IN BDV-INDUCED DAMAGE

The great variability in autistic conditions may be due to the complex mechanisms of the interaction between frank genetic mutation (e.g., Rett syndrome), unspecified contributions of genetic vulnerability, and/or environmental factors triggering abnormal brain development (London and Eztel, 2000; Lord et al., 2000). The BDV model allows us to study the pathogenic processes by which individual differences in responsiveness to environmental insults lead to variable autistic-like pathology and responses to treatment. Neonatal BDV infection leads to different brain pathologies, behavioral deficits, and responses to pharmacologic treatments in rat strains with greater or lesser vulnerability to environmental insults (Pletnikov et al., 2002b, 2002c). Specifically, compared to Lewis rats, Fisher344 rats were found to be more vulnerable to neonatal BDV

infection, with greater thinning of the cortex, more profound alterations in the monoamine brain systems, and, as a result, more behavioral deficits. Moreover, this genetic background-specific BDV-induced damage led to different responses to pharmacologic treatments in the two rat strains. For example, a selective serotonin reuptake inhibitor, fluoxetine, significantly inhibited hyperreactivity in neonatally BDV-infected Lewis rats but not in BDV-infected Fisher344 rats. In contrast, a selective agonist of the serotonin 1A receptor, 8-OH-DPAT, suppressed hyperactivity in BDV-infected Fisher344 rats without affecting hyperlocomotion in BDV-infected Lewis rats (Pletnikov et al., 2002c). Thus, these results have indicated the utility of the BDV model for studying the complex pathogenic mechanisms of the genetic background–environment interplay.

ROLE OF THE IMMUNE SYSTEM

A great deal of data point to immune disturbances in autism (see Chapter 27, this volume). As indicated earlier, neonatal BDV infection induces no inflammatory response in the brain (Hirano et al., 1983; Narayan et al., 1983; Carbone et al., 1991; Gosztonyi and Ludwig, 1995). Nonetheless, reactive gliosis in various brain regions has been noted (Carbone et al., 1991, Hornig et al., 1999; Sauder and de la Torre, 1999, Weissenbock et al., 2000; Zocher et al., 2000), indicating that nonclassical or endogenous brain immune mechanisms may mediate developmental injury. For example, activated glial cells can initiate an inflammatory cascade, leading to the production of inflammatory and regulatory cytokines and chemokines that, in turn, are capable of producing an array of pathologic changes, from moderately disturbed chemical neurotransmission to severe neuronal loss (Little and O'Callagha, 2001; Biber et al., 2002). Several groups have recently demonstrated BDV-associated up-regulation of mRNAs for regulatory and proinflammatory cytokines and chemokines in neonatally BDV-infected rats. Specifically, BDV-associated elevation was noted in the expression of mRNA encoding interleukin-6 (IL-6); tumor necrosis factor (TNF-α); interleukin-1α (IL-1α) and 1β(IL-1β); and TGF-β mRNA levels in the parietofrontal cortex, hippocampus, hypothalamus, and cerebellum from postnatal day 7 and up to 4.5 months postinfection (Hornig et al., 1999; Plata-Salamán et al., 1999; Sauder and de la Torre, 1999). In contrast, levels of mRNAs for IL-2; IL-3; IFN-γ; and Th_2-type cytokines IL-4, IL-5, and IL-10 remained unaffected by BDV infection (Hornig et al., 1999; Weissenbock et al., 2000). Increased expression of IP-10 and RANTES chemokine genes in neonatally BDV-infected rats has been also reported (Sauder et al., 2000). Thus, cytokines and chemokines may be important participants in mediating viral neurotoxicity and ensuing neuronal death (Little and O'Callagha, 2001; Biber et al., 2002).

Conclusion

Neonatal BDV infection has a number of similarities to different symptoms and features of autism, including brain pathology, neurochemical alterations, and behavioral deficits, and studies of this model can be used to guide pathogenetic studies in humans. Moreover, depending on the time, dose, and route of infection, as well as genetic background, sex, or host age, the BDV model can variously affect different systems in the body (e.g., the immune system, CNS, gastrointestinal tract) (Pletnikov et al., 2002a), potentially mimicking a complexity of clinical outcomes and variety of the pathogenic processes in autism (Lord et al., 2000). The future studies of this BDV model can include testing pathogenesis hypotheses generated by clinical studies and observations that will further validate the use of the BDV model for autism research. Preclinical testing of novel pharmacologic compounds is another major direction for future use of the BDV model. Besides studies with immediate clinical implications, future experiments could be aimed at elucidating molecular and cellular mechanisms of direct and indirect virus toxicity in mediating abnormal brain maturation. This line of investigations would be especially important for further understanding the role of infectious and immune factors in the general phenomenon of neurodevelopmental damage and associated behavioral deficits. Given host variability in the expression of a behavioral disease in BDV-infected animals, the BDV model could also be useful for studying the role of individual characteristics in determining differential susceptibility to environmental factors leading to variable abnormalities.

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Gene Expression in Autism

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The human genome is estimated to contain 35,000 genes (Lander et al., 2001). In all eukaryotic cells, the expression of a gene that encodes a protein proceeds in several steps: the gene is transcribed, the RNA product is processed and transported out of the nucleus, and the mature messenger RNA (mRNA) is translated into protein. Genes are regulated in their expression at different developmental stages, body regions, and physiologic states. For a variety of human diseases, gene expression varies as a function of a pathologic condition. This chapter reviews the role of gene expression in autism.

Gene expression changes in autism may be considered from two perspectives: the primary gene defect and the various secondary effects that occur in affected cells. The primary gene defects that cause autism are unknown, but it is conceivable that mutated, disease-causing genes are expressed at abnormally low levels. For example, a chromosomal microdeletion can cause haploinsufficiency, or a gene may have a nonsense mutation that destabilizes the corresponding mRNA. The gene or genes that are mutated in autism could be expressed primarily in the central nervous system, or they could be expressed peripherally. For many neurologic disorders, such as Huntington disease, Rett syndrome, and fragile X syndrome, the defective gene is expressed in various regions throughout the body.

Secondary effects on gene expression are likely to be pervasive. A mutation in one gene (or in several genes) may initiate a cascade of downstream changes that define the phenotype of the diseased cell. For example, Rett syndrome is a disease with symptoms including autistic behaviors. It is caused by mutations in the *MECP2* gene encoding methyl CpG binding protein 2, a transcriptional repressor (Amir et al., 1999). The consequence of *MECP2* mutations may be the failure of cells to repress thousands of genes. As another example of a disease with features of autism, fragile X syndrome is caused by mutations in a gene encoding fragile X mental retardation protein (FMRP), an RNA-binding protein.

A large number of mRNA transcripts from fragile X syndrome cells are abnormally regulated (see below).

The expression of a single gene may be measured using standard molecular biology techniques, such as Northern blotting or the polymerase chain reaction with reverse transcription (RT-PCR). The emerging technology of DNA microarrays allows the rapid, simultaneous, and quantitative profiling of thousands of transcripts from a biological sample (Hegde et al., 2000). Total RNA or mRNA is extracted from samples, such as biopsies, or cell lines from diseased and normal samples. The mRNA is typically converted to complementary DNA (cDNA) and then labeled with fluorescence or radioactivity and hybridized to solid supports containing immobilized cDNAs or oligonucleotides of known sequence (microarrays). After washing to remove nonspecifically bound probe, image analysis is used to quantitate signals corresponding to the expression of each gene represented on the microarray. Data analysis is then applied to define significantly regulated genes, as well as altered patterns of gene expression (Brazma and Vilo, 2000).

DNA microarray technology has been applied to the study of neurologic disorders, such as schizophrenia (Mirnics et al., 2000; Hakak et al., 2001; Hemby et al., 2002), multiple sclerosis (Whitney et al., 1999; Lock et al., 2002), Alzheimer disease (Ginsberg et al., 2000), brain tumors (Markert et al., 2001), cerebral ischemia (Jin et al., 2001), Rett syndrome (Colantuoni et al., 2001), and autism (Purcell et al., 2001b). These studies have been used to identify individual regulated genes, such as genes highly expressed in plaques from a multiple sclerosis patient (Whitney et al., 1999). Microarray studies have also been used to classify patients with neurologic disease from controls (Colantuoni et al., 2001), analogous to the clustering of cell lines or tumors from patients with cancers (Golub et al., 1999; Tamayo et al., 1999; Alizadeh et al., 2000; Bittner et al., 2000). The use of microarrays to assess gene expression in neurologic disorders has been reviewed (Colantuoni et al., 2000; Bahn et al., 2001; Greenberg, 2001; Shilling and Kelsoe, 2002).

Challenges in the Study of Gene Expression in Autism

Gene expression profiling of tissue samples from autistic individuals may be useful to identify significantly regulated genes. Such genes (and their corresponding protein products) could serve as markers for autism. Genes that are abnormally regulated in their expression levels could also reflect disturbances in a cellular pathway that is indicative of a molecular lesion. However, a number of methodologic questions are relevant to gene expression studies of autism (Purcell et al., 2001a).

Which samples are appropriate to study? There are three principal choices of biologic samples to measure: postmortem brain samples, peripheral cell lines, or tissues from animal models. Postmortem brain samples are available in only limited quantity and may suffer from long postmortem intervals, uncertain agonal states, and uncertain clinical histories, including medication history (Colantuoni et al., 2000). Lymphoblasts (Geschwind et al., 2001) and fibroblasts are readily available peripheral cell lines, but it is not known whether such cells exhibit changes in gene expression that are relevant to autism. For a variety of neurologic disorders that have autistic-like features, the gene responsible for the disease is expressed in peripheral cells. These diseases include Rett syndrome (Amir et al., 1999), fragile X syndrome (Bardoni et al., 2000), tuberous sclerosis (van Slegtenhorst et al., 1997), and succinylpurinemic autism (adenylosuccinase deficiency). Finally, animal models have been proposed for autism (Andres, 2002), such as Borna virus–exposed rats (Hornig et al., 2001) or primate models (Hemby et al., 2001). However, it is challenging to study animal models of such disorders as autism that affect higher cognitive functioning.

How heterogeneous is autism? Autistic disorder may represent a constellation of several distinct disorders in which affected individuals share common phenotypes. As many as 25 percent of patients diagnosed with autism present with other medical disorders, such as fragile X syndrome, tuberous sclerosis, and chromosomal abnormalities. Additionally, whole genome linkage scans suggest that the prototypic autistic disorder is likely to be caused by mutations in multiple genes (Lamb et al., 2000; Gutknecht, 2001). Thus, molecular studies of autism may be confounded by the heterogeneity of the disorder (Folstein and Rosen-Sheidley, 2001). A study of postmortem brain samples from 10 individuals with autism and 10 controls could thus involve brain material from several distinct diseases associated with a common phenotype. Even for normal (control) human brains, the variation in gene expression is poorly characterized (Saito-Hisaminato et al., 2002). The extent of variation of gene expression between brain regions and between individuals may be great, and gene expression may be influenced by such factors as undiagnosed conditions (e.g., mental illnesses, alcoholism) or drug effects.

Which developmental time periods are most relevant? Informative, relevant gene expression changes could occur in a critical time period of development (e.g., birth to age 5 years), whereas gene expression studies using postmortem brain typically involve samples from patients who died at ages 10–30 years. Thus, studies using samples from relatively older patients provide a view of the disease process at one particular stage. This can be useful for revealing the cellular and molecular responses to the initial insults that cause autism in early development.

Which brain regions are most relevant to study? Changes in gene expression could occur in a selected brain region. A gene with a mutation that causes autism could be expressed in only selected cell types or selected cell regions. As in the case of phenylketonuria, the defective gene product might even be expressed outside the brain while causing a neurologic phenotype. Studies of human brain typically rely on regions that have been implicated based on neuropathology studies.

Are the observed changes in gene expression due to primary or secondary causes? Any comparison of gene expression in brain samples from normal individuals versus those with autism is likely to result in the identification of differentially regulated genes. However, these differences could be accounted for by secondary effects, such as seizures, which are present in about one-third of all individuals with autism (Volkmar and Nelson, 1990), or mental retardation (present in ~75% of autistic individuals). Seizure-induced gene expression has been profiled in mouse hippocampus (French et al., 2001) and rat blood (Tang et al., 2001), and a description of such regulated genes may be useful in the analysis of gene expression in samples from patients with autism.

Gene Expression in Individuals with Autism

Gene expression in postmortem cerebellar samples from 10 individuals with autism was measured relative to age-, gender-, and regionally matched controls (Purcell et al., 2001b). This study employed two microarray platforms (based on the labeling of cDNA with radioactivity or fluorescence) and resulted in the identification of several genes that were differentially regulated. These included a modest degree of up-regulation of genes encoding phospholipase A_2, the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic-acid (AMPA) 1 glutamate receptor, and the excitatory amino acid transporter 1 (EAAT1). Down-regulated genes included those encoding somatostatin receptor 2 and histidine decarboxylase. Subsequent analyses of individual genes using RT-PCR confirmed that several genes encoding proteins associated with glutamatergic neurotransmission were up-regulated, including the types 1, 2, and 3 AMPA glutamate receptor and the glutamate transporters EAAT1 and EAAT2.

Even if a transcript is differentially regulated in autism, it is unclear whether there is a functional consequence in terms of regulation of the amount of the corresponding protein product. Several studies suggest a relatively poor correlation between individual mRNA and protein levels in yeast and mammalian systems (Gygi et al., 1999; Chen et al., 2002), although a positive overall correlation between mRNA levels and protein abundance has been reported (Greenbaum et al., 2002). Based on Western blotting, Purcell et al. (2001b) found that the levels of several proteins corresponding to regulated mRNA transcripts were significantly up-regulated in samples from autistic patients, including the AMPA1

receptor, EAAT1 and EAAT2, Band 4.1N (a protein that tethers glutamate receptors to the actin cytoskeleton), and the glutamate receptor interacting protein GRIP1. These changes were not related to seizures or antiseizure medications taken by the subjects with autism. A decreased number of AMPA-type receptors was detected by glutamate receptor autoradiography of cerebellar sections using [^{3}H]AMPA as a ligand. Increased mRNA encoding proteins that function in glutamatergic neurotransmission could represent a compensatory response to a functional down-regulation of this pathway.

Investigators have examined the roles of a variety of other proteins in autism, although few studies have focused on gene expression. Transcriptional profiling using microarrays provides data on the expression of thousands of genes, allowing the subsequent follow-up study of a limited number of differentially regulated genes. Other approaches have been taken to assess the role of individual genes in autism. A variety of candidate genes have been selected based on genetic studies (such as linkage and association studies, or studies of regions of chromosomal rearrangements in patients with autism) or on the behavioral phenotype associated with the loss of a gene's function. Examples of proteins that have been studied are as follows. Wassink and colleagues (2001b) examined *WNT2* as a candidate gene and described its normal expression in the thalamus. *Reelin,* which like *WNT2,* is localized to the long arm of chromosome 7, has been evaluated as a candidate gene in autism (Persico et al., 2001). Fatemi and colleagues (2001) reported reduced levels of this protein and Bcl-2 in postmortem brain samples relative to normal controls. Pliopylys and colleagues (1990) reported a significantly decreased level of neural cell adhesion molecule (NCAM) in serum samples of patients with autism, but Purcell and colleagues (2001c) reported no significant change in NCAM protein or mRNA levels in serum and brain samples. Blatt and colleagues (Blatt et al., 2001) observed decreases in gamma-aminobutyric acid (GABA) receptors in the brains of individuals with autism, based on receptor autoradiography studies. Notably, they did not detect changes in glutamate or several other neurotransmitter receptor systems. Perry and colleagues described both increases and decreases in cholinergic receptor subtypes by ligand autoradiography, Western blotting, and immunohistochemistry (Perry et al., 2001).

Gene Expression in Autism-Related Disorders

Fragile X Syndrome and Rett Syndrome

In addition to studies on patients diagnosed with autism, gene expression has been assessed in disorders that sometimes include features of autism. The relevance of gene expression studies of fragile X syndrome, Rett syndrome, and chro-

mosome 15 disorders to autism will not be known until genes mutated in autism are identified.

Fragile X syndrome (MIM 309550) is a form of X-linked mental retardation associated with a deficiency of the fragile X mental retardation protein (FMRP). This deficiency is caused by a $(CGG)_n$ trinucleotide repeat expansion in the 5′ untranslated region in the *FMR1* gene encoding FMRP. *FMR1* has two paralogs (*FXR1* and *FXR2*), which encode homologous RNA binding proteins. The phenotype of fragile X syndrome includes autistic-like behavior in about 25 percent of the patients (Bailey et al., 1998). Although these are distinct disorders, some patients initially diagnosed with autism have fragile X syndrome (Wassink et al., 2001).

In fragile X syndrome cells, some mRNAs may fail to bind *FMRP* or its two paralogs, contributing to the phenotype of the disorder. D'Agata and colleagues (2002) identified mRNAs associated with *FMRP* by immunoprecipitating the protein from mouse brain and identifying bound mRNAs using microarrays (Brown et al., 2001). A comparison of immunoprecipitated mRNAs between wildtype and *FMR1* knockout mouse brain resulted in the identification of several hundred candidate genes. Additionally, they isolated human mRNA from normal or fragile X mutant lymphoblastoid cell lines and used microarrays to identify regulated genes. Subsets of these genes were derived from a high molecular weight polyribosome fraction of fragile X cells. This study is useful in its identification of candidate genes that may be responsible for the pathophysiology of fragile X syndrome.

Rett syndrome is a progressive neurodevelopmental disorder that primarily affects girls. It is characterized by microcephaly, seizures, loss of speech and of purposeful hand movements, and autistic-like behaviors. The disease is caused by mutations in an X-linked gene encoding methyl CpG binding protein 2 (*MeCP2*) (Amir et al., 1999). *MeCP2* is a transcriptional repressor that recruits histone deactylase and other proteins to alter chromatin structure. It is possible that mutations in the *MECP2* gene lead to a global failure to repress transcription of many genes. In an effort to identify these genes, Colantuoni and colleagues (2001) measured gene expression in postmortem Rett syndrome brain samples and matched controls. They identified a group of significantly regulated genes, including genes that are potentially differentially regulated as a consequence of *MECP2* mutations. They also reported that genes encoding neuronal proteins tended to be down-regulated, whereas genes encoding glial proteins tended to be up-regulated.

GENOMIC IMPRINTING

A variety of chromosomal abnormalities are associated with autism. Some of these involve gene dosage imbalance, and thus, the disease mechanism could

involve alterations in the normal levels of gene expression. About 1 percent of patients diagnosed with autism have an interstitial duplication of chromosome 15q11–q13. This region is of particular interest because it is subject to genomic imprinting, a phenomenon in which genes are expressed depending on their parental origin. Most genes are expressed from either the maternal or paternal allele; for several dozen genes that undergo genomic imprinting, one allele is selectively silenced. Microdeletions of 15q11–q13 can cause Prader-Willi syndrome, a contiguous gene-deletion syndrome caused by the loss of genes that display paternal expression (Nicholls and Knepper, 2001). Angelman syndrome is caused by defects in the maternally derived imprinted region of chromosome 15q11–q13 (Nicholls and Knepper, 2001). This syndrome is sometimes caused by mutations in a gene in this region, the maternally expressed *UBE3A* gene encoding E3 ubiquitin protein ligase. Duplications of 15q11–q13 are associated with autistic phenotype and/or pervasive developmental disorder (Bolton et al., 2001), although mutations in *UBE3A* were not identified in a screen of karyotypically normal autistic individuals (Veenstra-VanderWeele et al., 1999).

In addition to the 15q11–q13 region, a variety of other chromosomal regions have been implicated in autism. Skuse (2000) suggested that an imprinted gene on the X chromosome could account for the high male:female sex ratio observed in autism. Imprinting can be tissue-specific, adding to the phenotypic complexity of this process in causing human disease (Weinstein, 2001).

Conclusion

The global measurement of gene expression in tissue or cell samples from individuals with autism is now possible using DNA microarray technology. However, this approach is beset by a variety of technical challenges, due to the complexity of both the human brain and autistic disorder. It will be especially difficult to identify genes that are selectively up- or down-regulated in their expression levels as a primary function of autism. Such secondary effects as seizures or mental retardation associated with autism may be associated with changes in gene expression.

There are two main approaches to the analysis of gene expression data. First, inferential statistics can be used to define genes that are significantly related in samples from autistic individuals. Such tests may result in the identification of novel markers for autism. Regulated genes may also suggest biochemical pathways that are important in the pathophysiology of autism. Second, exploratory statistics may be used to classify groups of individuals on the basis of gene expression through clustering, principal components analyses, or other data visualization techniques. Although autism is a heterogeneous disorder, clustering could define patient subpopulations.

A premise of these approaches is that gene expression differences are associated with the condition of autism, but this is not certain. It is also not known whether abnormal mRNA levels are associated with changes in the levels of the corresponding protein products. In a study of 10 cerebella from postmortem autism cases, a variety of transcriptional changes in the glutamatergic neurotransmitter system were independently confirmed at the protein level by Western blot analysis (Purcell et al., 2001b).

A variety of evidence suggests that autism is caused by mutations in one or more genes. To date, no genetic mutations have been identified for cases of classically defined autism. Assuming that mutations are identified in the future, more focused gene expression profiling experiments may be undertaken. In the cases of fragile X syndrome and Rett syndrome, transcriptional profiling using microarrays has led to the identification of molecules that may be directly relevant to the function of the mutated gene product. Studies such as these allow the generation of experimental hypotheses about the molecular defect that underlies autism and autism-related disorders.

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16

Candidate Susceptibility Genes for Autism

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Autism is a pervasive developmental disorder that affects approximately one child in every 2,000 with a male:female ratio of 4:1 (Fombonne, 1999). The primary characteristics of autism were first described by Leo Kanner (1943) as an inability to relate to people and situations in an ordinary way, stereotyped movement, normal cognitive potential, and excellent rote memory. Currently, autism is recognized as a complex neurodevelopmental disorder characterized by the inability to establish and maintain relationships with others and impairment in nonverbal and verbal communications, accompanied by delay or lack of language, stereotyped behavior, repetitive movements, and restricted interests (Bailey et al., 1996). Autism is often accompanied by epilepsy and mental retardation. The onset of the disorder is within the first 3 years of life, and diagnosis is based mainly on the observation of behavioral dysfunctions and abnormal development of language. The spectrum of clinical manifestations and the severity of the disorder are variable, and they differ significantly even among siblings (Bailey et al., 1996; Happe and Frith 1996; Maestrini et al., 2000). Occasionally, autism overlaps with fragile X syndrome (August and Lockhart, 1984; Feinstein and Reiss, 1998), tuberous sclerosis (Smalley et al., 1992), or Angelman syndrome (Williams et al., 2001), further complicating diagnosis.

Genetic Factors

The possible contribution of genetic factors to autism as "inborn autistic disturbances of affective contact" (Kanner, 1943) is now confirmed. Family studies show that the disorder is more frequent in families with autistic probands (Ritvo et al., 1989), and the autistic phenotype is also less variable in relatives (Spiker et al., 1994). The detected risk of recurrence is approximately 75 times higher between sibs than in the general population (Bolton et al., 1994).

Evidence for genetic predisposition to autism also comes from twin studies. Folstein and Rutter (1997) showed different concordance rates between monozygotic (MZ) and dizygotic (DZ) twins, and found that in most sib pairs discordant for autism, the disorder was caused by brain injuries. Bailey et al. (1995) confirmed these data and showed 92 percent concordance for social and cognitive abnormalities in MZ twins and 10 percent concordance in DZ twins. Studies of twins performed by Pickles et al. (1995) and Greenberg et al. (2001) also provide evidence for a genetic basis of autism. The heritability of autism is estimated as 90 percent (Szatmari et al., 1998).

Several studies describe individuals with autism or behavioral features of autism caused by different chromosomal abnormalities (Gillberg, 1998; Wassink et al., 2001a). Although these cases are rare, they provide additional evidence for a genetic component in the disorder.

Difficulties in Locating Autism Susceptibility Loci

The genetic mechanism of autism is unknown. Unlike single-gene disorders, in which a mutation in one gene leads to a pathologic phenotype and is inherited within the family, autism is thought to be a multiple gene disorder of unknown mode of inheritance, with several interacting genes of moderate effect on different chromosomes (Jorde et al., 1991). The difference in concordance rates between MZ and DZ twins supports this observation (Bailey et al., 1995). As estimated by Risch et al. (1999), as many as 15 different genes of moderate effect could be involved in the disorder. Genome-wide linkage studies for autism (for reviews, see Maestrini et al., 2000; Buxbaum et al., 2001; Liu et al., 2001) suggest several susceptibility loci on different chromosomes. To date, autism susceptibility loci implicate almost every chromosome. However, none of these regions show highly significant evidence for linkage (Li et al., 2002).

Based on reported associations between biologic functions of genes and autism phenotypes, several positional candidates were studied in autistic individuals. Found variants were genotyped in additional autistic individuals and controls and analyzed for association with the disorder. Some of the variants showed positive association with autism in the samples studied (Table 16.1).

Chromosome 7

Studies indicate that chromosome 7q harbors an autism susceptibility gene. Several groups have reported linkage to different loci at 7q31–33 as the result of whole-genome scans (International Molecular Genetic Study of Autism Consortium, 1998, 2001; Barrett et al., 1999; Philippe et al., 1999; Risch et al., 1999).

TABLE 16.1. Candidate Susceptibility Genes in Autism

Gene	*Chromosome*	*Variant*	*Pos +/Neg–*	*Reference*
GRIK2	6q16.3–q21	(TAA) repeat D6S283 D6S1543 M867I	+	Jamain et al. (2002)
WNT2	7q31.31	Arg299Trp Leu5Arg C783T	+ –	Wassink et al. (2001b) McCoy et al. (2002)
RELN	7q22.1	(GGC) repeat	+	Persico et al. (2001)
EN2	7q36.2	MP4	+	Petit et al. (1995)
HOXA1	7p15.2	A218G	+ –	Ingram et al. (2000) Li et al. (2002)
HRAS	11p15.5	3′VNTR	+	Herault et al. (1993) Herault et al. (1995) Comings et al. (1996)
AVPR1A	12q14.1	$(CT)_4$-TT-$(CT)_8$-$(GT)_{24}$	+	Kim et al. (2002b)
GABRB3	15q11–q13	155CA-2	+	Cook et al. (1998) Buxbaum et al. (2002)
GABRG3	15q11–q13	539T/C 687T/C	+	Menold et al. (2001)
UBE3A	15q11–q13	D15S122	+	Nurmi et al. (2001)
SLC6A4	17q11.1–q12	5-HTTLPR VNTR HTT SNP9 HTT SNP_{II}	+	Klauck et al. (1997) Cook et al. (1997) Yirmiya et al. (2001) Tordjman et al. (2001) Kim et al. (2002a)
NF1	17q11.2	GXAlu	+ –	Mbarek et al. (1999) Plank et al. (2001)
HOXB1	17q21.32	(ACAGCGCCC) insertion	+ –	Ingram et al. (2000) Li et al. (2002)
ADA	20q13.12	Asp8Asn	+	Persico et al. (2000) Bottini et al. (2001)

Chromosomal rearrangements in this region in autistic individuals have also indicated the presence of an autism-related gene (Ashley-Koch et al., 1999; Sultana et al., 1999; Vincent et al., 2000; Warburton et al., 2000; Tentler et al., 2001). The 7q31–33 locus is also associated with speech and language abnormalities (*SPCH1*) (Lai et al., 2000), which are among the main features of autism. A recently identified forkhead-domain (*FOXP2*) gene, located at 7q31.1, was found to be *SPCH1,* mutated in a large family with severe speech and language impairment (Lai et al., 2001). No mutations in *FOXP2* were found in individuals with autism (Meaburn et al., 2002; Newbury et al., 2002).

Several other genes at 7q were analyzed in autistic individuals. Polymorphic variants in some of them showed a possible association with the disorder in the families tested. One of these genes is wingless-type MMTV integration site family member 2 (*WNT2*), located near the chromosomal breakpoint, which was found in one autistic patient (Vincent et al., 2000). This gene is a member of a family of WNT genes involved in development of the central nervous system (CNS). The coding sequence of *WNT2* was screened for mutations in 135 autistic individuals and 160 controls (Wassink et al., 2001b). Two different variants were detected in affected siblings and one parent in two different families. The variants found were the transition in exon 5 (Arg299Trp) and the transversion in exon 1 (Leu5Arg). Both variants occurred in functionally significant regions of the gene and changed the charge of conserved amino acids. These variants were not found in unaffected siblings and control subjects. The authors hypothesized that the variants in the *WNT2* gene could increase susceptibility to autism, although they cannot exclude the existence of a more common allele. Two other polymorphisms were also found in *WNT2:* C519T transition in 5′UTR and C783T transition in 3′UTR. Both polymorphisms were genotyped in autistic patients. The 5′UTR polymorphism was not in linkage disequilibrium with the disorder, whereas the T allele in 3′UTR was preferably transmitted to affected offspring, particularly those who had not developed phrase speech by 36 months. Another group of authors failed to reveal linkage disequilibrium with the same and other markers in the 3′UTR region of *WNT2* in replicating the study (McCoy et al., 2002). The ethnic origins of sample sets used in these two studies were the same. There were also no differences in allele frequencies of single nucleotide polymorphisms (SNP) between these two data sets. Finally, both the Arg299Trp and Leu5Arg mutations found by Wassink et al. (2001b) were not detected by secondary structural content prediction and denaturing high-performance liquid chromatography in the second study. McCoy et al. (2002) concluded that the *WNT2* gene is not a genetic risk factor for autistic disorder.

The other gene of interest on chromosome 7q is Reelin (*RELN*), which plays an important role in neural development. Recently it was found that the level of

Reelin protein is changed in the blood of patients with schizophrenia, major depression, and bipolar disorder (Fatemi et al., 2001a). It was also shown that the level of Reelin is decreased in the cerebellum of autistic individuals (Fatemi et al., 2001b). The deletion of the Reelin gene in mice causes developmental alterations, which are similar to those in the brains of autistic patients (Bailey et al., 1998). Because the gene is located at 7q22.1 (DeSilva et al., 1997), in a region still encompassing positive signals from several studies (International Molecular Genetic Study of Autism Consortium, 1998; Phillippe et al., 1999), it was analyzed for mutations in patients with autism. Several polymorphisms were identified, including an A/G transversion near the 5′ splice site of exon 6, and a T/C transversion in exon 50 (Persico et al., 2001). The most interesting one was the polymorphic GGC repeat located immediately 5′ of the ATG initiation codon of the Reelin gene. The number of repeats ranged from eight to 14 in the majority of samples, with a preponderance of shorter repeat alleles in the general population. A case-control association study showed that the longer alleles (11 repeat units or more) were shared by autistic individuals more than twice as often as by controls. A transmission disequilibrium test (TDT) of 172 families with one autistic child and both parents confirmed the preferential transmission of the longer repeat alleles to autistic offspring. Case-control and family-based haplotype analyses also showed preferential transmission of haplotypes surrounding longer alleles. It was hypothesized that transmission of "long" alleles from a parent enhanced the probability of a child being affected. It is possible that the GGC repeat is in linkage disequilibrium with a causative mutation. In this case, identification of additional polymorphisms in the 5′ region of the gene would help. To date, the demonstrated preferential transmission of longer GGC alleles and encompassing haplotypes to autistic individuals places the Reelin gene among genes potentially conferring a vulnerability to autistic disorder.

The role of other putative autism susceptibility genes on chromosome 7q has also been studied. The main criteria for choosing these genes were functional features of encoding proteins and their role in the development of cerebral structures. Several studies reported neuroanatomic abnormalities in the CNS of autistic patients, particularly in the cerebellum and brainstem (Tanguay et al., 1982; Courchesne et al., 1988; Bauman, 1991). One of the genes involved in cerebellar development is the engrailedlike homeobox (*EN2*) gene expressed in the cerebellum and mapped to chromosome 7q36.2 (Logan et al., 1989; Poole et al., 1989; LeRoux et al., 1993). The orthologous gene codes for one of the main proteins of the development pathways in *Drosophila* (Logan et al., 1992). Partial deletion of the mouse ortholog *En-2* caused abnormal foliation in the adult cerebellum (Joyner et al., 1991). Two polymorphisms of the human *EN2* gene were used for association studies in 100 unrelated children with infantile autism and 100

controls (Petit et al., 1995). The first polymorphism was identified in the 1.7-kb SstI genomic fragment (PvuII, MP4 probe) from the 5′ region of the gene (Logan and Joyner, 1989a), and the second one was found in the 1.2-kb PstI/TaqI fragment (SstI, MP5 probe) starting in the homeobox and extending to the 3′ region of the gene (Logan and Joyner, 1989b). Southern blot hybridization of genomic DNA from autistic individuals and controls with both MP4 and MP5 probes detected a significant difference in the allele distribution ($p < 0.01$).

Another gene on chromosome 7p15.2, *HOXA1,* and its paralog *HOXB1* (17q21.32) were also analyzed for mutations in patients with autism (Ingram et al., 2000; Li et al., 2002). Both genes regulate early development in vertebrates (Gehring, 1985). Of interest were anatomic anomalies in autistic brainstem and physical anomalies (malformed ears and hearing deficits) in autism caused by exposure to thalidomide (Rodier et al., 1997). These anomalies were similar to those in *Hoxb1* knockout mice (Mark et al., 1993, Strömland et al., 1994). These mice also showed a deficit of facial nucleus neurons and neurologic dysfunctions in the facial muscles (Goddard et al., 1996). A transition in the first exon of human *HOXA1* gene, and a 9-bp insertion and two transitions in the first exon of *HOXB1* gene were detected (Ingram et al., 2000). The transition in *HOXA1* segregated in 50 families with autism, and an interaction between *HOXA1* and *HOXB1* was proposed. Li et al. (2002) sequenced coding regions and splice junctions of both genes in 24 autistic individuals and found the same sequence variants. The A218G transition in *HOXA1* and 9-bp insertion in *HOXB1* were also genotyped in 110 multiplex families with autism (Li et al., 2002). The data were analyzed using the transmission disequilibrium test. Because no evidence of association with the disorder was found, the authors concluded that these two genes are unlikely to contribute susceptibility to autism in these families.

Although a strong candidate gene for susceptibility to autism on chromosome 7q has not been identified, several recent studies support an assumption of the presence of such a gene in this region (Bradford et al., 2001; Alarcon et al., 2002; Badner and Gershon, 2002).

Chromosome 15

Chromosomal aberrations in autism have been revealed in many chromosomes (Gillberg, 1998). Localization of deletions, inversions, translocations, and other rearrangements in autistic individuals could be very useful for mapping and identifying disease genes. Chromosomal duplications of maternal origin on chromosome 15q11–q13 were described as the most common chromosomal abnormalities among autistic individuals (Browne, 1997; Cook et al., 1998; Schroer et al., 1998), although no highly evident genotype-phenotype correla-

tions between duplications on chromosome 15q and autism have been found so far. Linkage to 15q11–q13 was demonstrated in one of the whole genome screens described earlier (Collaborative Linkage Study of Autism, 1999). Other groups also detected linkage and linkage disequilibrium at different markers in this region (Cook et al., 1998; Bass et al., 2000; Martin et al., 2000). Genes within this interval are considered to be potential candidates for genes underlying autism.

The 15q11–q13 interval contains the gamma-aminobutyric acid (GABA)-A receptor complex, consisting of at least 15 different genes (Glatt et al., 1997). GABA is the major inhibitory neurotransmitter in the brain, and it acts via the GABA-A receptors. Cook et al. (1998) analyzed one of these genes (GABA-A receptor beta 3 subunit gene, *GABRB3*) in 140 singleton families, and found association of the autistic phenotype with the marker 155CA-2 in the third intron of the gene. However, the closest flanking markers, D15S97 and D15S156, did not show any association in the same families. Two other groups (Maestrini et al., 1999; Martin et al., 2000) analyzed the same gene with the same markers in other families with autism and found no evidence for linkage or association. Association was detected by Martin et al. (2000) with only one marker, 60-kb centromeric to *GABRB3*. Recently, Buxbaum et al. (2002) performed an association study with 155CA-2 using the transmission disequilibrium test in a set of 80 autism families and confirmed the association detected by Cook et al. (1998) between autistic disorder and 155CA-2. *GABRG3* is another GABA-A receptor subunit gene in the same interval. An association between markers in exon 5 and intron 5 of this gene and autistic phenotype was found in 226 families using the pedigree disequilibrium test (Menold et al., 2001). These findings indicate that GABA-A receptor subunit genes should be analyzed further for susceptibility to autistic disorder.

Another interesting candidate gene in the 15q11–q13 interval is ubiquitin-protein ligase E3A (*UBE3A*). Preferential maternal expression in the human brain was detected for this gene in several studies (Albrecht et al., 1997; Vu and Hoffman, 1997). Transgenic mice with maternal Ube3a deficiency demonstrated motor dysfunction, inducible seizures, and a context-dependent learning deficit (Jiang et al., 1998). In mice with partial paternal disomy encompassing Ube3a, lack of maternal expression of Ube3a in the hippocampus and cerebellum was observed (Albrecht et al., 1997). The phenotype of these mice also correlates with phenotypic features of Angelman syndrome. Some phenotypic features of patients with Angelman syndrome are similar to those in autistic children (stereotyped behavior, seizure disorder, ataxia), although most of them can be distinguished from autism after clinical evaluation (Williams et al., 2001). Mutations in patients with Angelman syndrome have been found in the *UBE3A* gene (Laan et al., 1999). This gene has also been considered for a positional and functional candidate in autistic disorder, associated with duplications in the

region 15q11–q13. Attempts to detect linkage disequilibrium between *UBE3A* and four markers inside the gene did not reveal a significant linkage (Cook et al., 1998). The *UBE3A* gene was also analyzed for sequence variations in 10 unrelated individuals with autism (Veenstra-VanderWeele et al., 1999). Although four known and three new, probably nonfunctional, polymorphisms were detected, no mutations disrupting the expression of *UBE3A* gene have been found. Recently, several markers across *UBE3A* and the GABA-A complex were analyzed for linkage disequilibrium in 94 sib-pair families with autism, and significant linkage was revealed with the D15S122 marker, located in the 5′ untranslated region of the *UBE3A* gene (Nurmi et al., 2001). A maternal effect was also observed for preferential transmission of one of the alleles at this marker. Although no functional mutations were detected in the coding region and putative promoter of the gene, the potential involvement of *UBE3A* in autistic disorder should be further investigated.

Other Chromosomes

In one genome scan, Philippe et al. (1999) identified an autism susceptibility locus on chromosome 6. Significant linkage to 6q was found at the marker D6S283. Recently, additional markers in the region were genotyped (Jamain et al., 2002). The most significant LOD scores were obtained for the marker D6S1543 in intron 1 and the polymorphic TAA repeat in exon 16 of the glutamate receptor 6 (*GRIK2*) gene. Using three SNPs in intron 14, exon 15, and exon 16 of *GRIK2,* family-based linkage and association analyses were performed in the same families and in a new set of more than 100 parent-offspring trios. TDT analysis showed a significant association of the *GRIK2* gene with autistic disorder ($P = 0.008$). Both findings suggest linkage between *GRIK2* and autism. Sequencing of the *GRIK2* gene in more than 30 autistic individuals revealed several new polymorphisms. One of them (M867I) caused a change of methionine to leucine in a highly conserved region of the encoded protein. The leucine allele was found to occur more frequently in autistic individuals than in controls. The *GRIK2* gene codes for glutamate—one of the major excitatory neurotransmitters of the central nervous system, which interacts with several glutamate receptors (Ziff, 1999). The gene is widely expressed in those regions of the brain involved in learning and memory (Dingledine et al., 1999). Altered levels of glutamate were found in the platelets and plasma of autistic individuals (Rolf et al., 1993; Moreno-Fuenmayor et al., 1996). It was also hypothesized that infantile autism is a hypoglutamatergic disorder (Carlsson, 1998). This evidence makes the glutamate receptor 6 gene a good candidate for an autism susceptibility locus.

Associations of autism with several other genes have been reported. One of these genes is the serotonin transporter (*SLC6A4*) on chromosome 17q11.1–q12. Possible involvement of *SLC6A4* in autistic disorder is based on several observations. It was reported that serotonin transporter inhibitors reduce repetitive behavior and thoughts in adults with autistic disorder (McDougle et al., 1996). Lesch et al. (1996) found association of a polymorphism in the promoter region of the gene with anxiety, which has increased incidence in families with autistic individuals (Piven et al., 1991). It was also proposed that the serotonin transporter plays a role in platelet hyperserotonemia in autism (Anderson et al., 1990; Cook et al., 1993). Evidence for linkage at *SLC6A4* was obtained in a whole genome scan by the International Molecular Genetic Study of Autism Consortium (2001). Although all other genome scans did not reveal any evidence for linkage at this locus, the important biological role of the 5-serotonin transporter in the brain made *SLC6A4* a candidate gene for susceptibility to autism. Two polymorphisms in this gene, a 44-bp deletion/insertion in the promoter region (5-HTTLPR) and a variable number tandem repeat (VNTR) in intron 2, were used for determination of preferential allele and haplotype transmission in families with autistic disorder. Several studies reported preferential transmission of the long (Klauck et al., 1997; Tordjman et al., 2001; Yirmiya et al., 2001) or the short (Cook et al., 1997) alleles of 5-HTTLPR. In one of the recent studies, Kim et al. (2002a) identified more than 20 novel SNPs and several polymorphic microsatellites after sequencing *SLC6A4* gene and its flanking regions in 10 autistic individuals and 10 controls. A dense marker map was constructed. All the new markers and both 5-HTTLPR and VNTR polymorphisms were analyzed in 81 trios by TDT. Transmission disequilibrium was found for the 5-HTTLPR/VNTR haplotype and for nine markers: 5-HTTLPR, VNTR, and seven SNPs. The researchers obtained significant evidence for two of these SNPs (one in the 5′ flanking region of exon 1B of *SLC6A4* and the other in exon 12 of the cysteine protease bleomycin hydrolase [*BLMH*] gene). These data provide additional support for association between *SLC6A4* and autistic disorder.

Association of the Asp8Asn polymorphism, in the adenosine deaminase gene (*ADA*), with autistic disorder has recently been observed in three Italian populations (Persico et al., 2000; Bottini et al., 2001). Adenosine deaminase is an enzyme that plays an important role in regulation of adenosine levels, a very important factor for the development and function of the nervous and immune systems. Adenosine is involved in the regulation of serotonin transmission, which showed some abnormalities in children with autism (Cook and Leventhal, 1996). Several sequence variants in this gene have also been found in patients with immunodeficiency disease (Hirschhorn, 1993). The most common variant is the Asp8Asn polymorphism. The presence of the Asn8 allele led to the

expression of the ADA enzyme with reduced enzymatic activity (Hirschhorn et al., 1994). Because some children with autistic disorder demonstrate reduced activity of the ADA enzyme in serum (Stubbs et al., 1982) and other changes in purine metabolism (Page and Coleman, 1998), the possible role of Asp8Asn polymorphism was investigated. Case-control studies performed by Persico et al. (2000) and Bottini et al. (2001) indicated that the Asn8 allele of adenosine deaminase is more than twice as frequent in autistic individuals as in controls. A follow-up two-loci haplotype analysis of autistic and normal individuals confirmed the hypothesis that the only Asn8 variant inside the *ADA* gene may influence susceptibility to autistic disorder (Lucarelli et al., 2002). Although linkage to chromosome 20q13, containing the *ADA* gene, was not suggested by any of whole-genome scans, a possible role of the ADA enzyme in the autistic phenotype cannot be excluded at this time.

In one recent study, Kim et al. (2002b) obtained evidence for linkage between one of the polymorphisms in the arginine vasopressin receptor 1A (*AVPR1A*) gene, located on chromosome 12q14.1. Based on the observation that transgenic mice with a repetitive sequence in the 5′ region of the *AVPR1A* gene demonstrate increased affiliative behavior after injection with arginine vasopressin when compared to mice lacking this repetitive region (Young et al., 1999), the authors hypothesized that mutations in the *AVPR1A* gene could influence social behavior in humans. Two polymorphisms located 3.6 kb and 0.5 kb upstream of the transcription start site of the gene were genotyped in 115 autism trios and analyzed for transmission disequilibrium with autism by the multiallelic transmission disequilibrium test. Evidence for transmission disequilibrium was obtained with one of the polymorphisms, located 3.6 kb from the coding region of the gene. It is still not clear if this polymorphism is in linkage disequilibrium with another variant, or if it influences expression of the human *AVPR1A*.

Association between autism and an allele in the c-Harvey-Ras (*HRAS*) gene has been reported in two small population-based association studies. However, in each case, the statistical evidence was weak: $P = 0.05$ (Herault et al., 1993, 1995), and $P = 0.04$ (Comings et al., 1996). Mbarek et al. (1999) proposed an association between autistic disorder and the neurofibromatosis type 1 (*NF1*) gene on chromosome 17q11.2 in 85 patients from France. But the same marker in the *NF1* gene showed a lack of association in more than 200 autistic individuals from South Carolina (Plank et al., 2001). Involvement in autistic disorder has also been hypothesized for the GABA transaminase (*GABA-T*) gene on chromosome 16p13.3 (Cohen, 2001) and the syndecan-2 (*SDC2*) and gastrin-releasing peptide receptor (*GRPR*) genes on chromosomes 8q22–q24 and Xp22.13, respectively (Ishikawa-Brush et al., 1997), as well as genes of the major histocompatibility complex, MHC (Torres et al., 2001).

Conclusion

To date, positive associations with autism have been reported for more than 15 genes residing in different loci of human chromosomes. Analysis of the candidate genes usually followed genome-wide linkage studies. Some genes were analyzed based on their proposed functions. Positive findings for several genes (*GABRB3, SLC6A4,* and *ADA*) have been replicated and confirmed in independent samples. For others (*WNT2, HOXA1, HOXB1,* and *NF1*), replication efforts were unable to confirm initial findings (see Table 16.1).

Sequence variations were often detected as common variants not only in autistic, but also in nonautistic individuals (Mbarek et al., 1999; Kim et al., 2002a). The high frequency of these variants may create problems for identification of disease-susceptibility alleles (Antonarakis et al., 2000), although high-frequency alleles are assumed to be exactly those genetic factors that underlie complex disorders (Lander, 1996; Chakravarti, 1999). Variations may also arise in carriers who do not express an autistic phenotype due to incomplete penetrance, or lack of interaction between mutated genes. Another problem could be genetic heterogeneity, in which common variants arise in two or more genes, leading to the same phenotype (Loughlin et al., 2002; Paloneva et al., 2002; see also Lander and Schork, 1994). Further studies with larger sample sizes and perhaps phenotypically homogeneous subgroups (e.g., see Buxbaum et al., 2001) should lead to the identification of autism susceptibility genes.

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17

Chromosome 15 and Autism

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As covered in Chapter 18, autism is considered a strongly genetic disorder. Heterogeneity is prominent in autism, as in most clinical syndromes. One aspect of heterogeneity relevant to autism genetics is the presence of chromosomal anomalies, and several chromosomal anomalies have been found in patients with autism spectrum disorders (ASD) that involve chromosome 15. In addition, linkage and linkage disequilibrium studies relevant to chromosome 15q reveal some evidence for one or more susceptibility variants, particularly in the 15q11–q13 region overlapping most of the chromosomal anomalies involving chromosome 15 found in autism.

Chromosome 15 Anomalies with Increased Risk of Autism

Although the most common chromosomal anomalies found in samples of patients with autism are cases in which one or more extra copies of 15q11–q13 region are present (Cook et al., 1997; Schroer et al., 1998; Weidmer-Mikhail et al., 1998; Wolpert et al., 2000; Wassink et al., 2001; Estecio et al., 2002), more is known about two syndromes in which the classic cases are defined by deletion of the same region: Angelman syndrome (AS) and Prader-Willi syndrome (PWS). Given that these syndromes are better understood than duplication 15q11–q13 syndromes, they will be covered first. An increased risk of autism is also present in persons with Angelman syndrome or Prader-Willi syndrome.

ANGELMAN SYNDROME

AS is characterized by severe hypotonia, epilepsy, mental retardation ranging from profound to moderate in severity, ataxia, and paroxysymal laughter. Most cases (~70%) of AS are due to de novo deletion of an approximately 4-megabase

(Mb) region that is among the most proximal euchromatin (containing expressed genes) on the long arm of chromosome 15 (15q11–q13). (Note that the most proximal band on each chromosome's long arm is q11.) A smaller number of cases have paternal uniparental disomy (UPD), in which two otherwise normal copies of chromosome 15 come from the father and none from the mother. Although frequencies of less than 2 percent have been reported for UPD in AS, this may be due to more severe phenotype in deletion cases and selective screening of patients with AS with more severe phenotype. For example, a study in which a large number of patients with AS were screened revealed a similar number of patients with AS due to deletion and UPD (Jacobsen et al., 1998). Imprinting refers to the silencing of a parent of origin-specific copy of a gene in some tissues at some point in development. This suggests that the absence of expression of genes that are active only when inherited from the mother in some tissues contributes to the pathogenesis of AS.

The third mechanism leading to AS is imprinting mutation. Imprinting mutations interrupt the normal resetting of the imprint. Instead of resetting the imprint of a chromosome inherited from the maternal grandfather, the mother passes on a chromosome with a paternal instead of maternal imprint. Therefore, even though there is no deletion of 15q11–q13 and one chromosome is inherited from each parent, the functional equivalent of paternal UPD results in AS.

The first three classes of AS may be identified by a methylation blot in which there is a deletion of the maternal fragment on Southern blot after digestion with restriction enzymes that specifically cut maternal and paternal genomic fragments. However, the fourth class of AS is due to a mutation of *UBE3A,* the gene encoding ubiquitin protein ligase E3A (Matsuura et al., 1997). Interestingly, in mouse, *Ube3a* is expressed solely from the maternal allele only in the hippocampus, Purkinje cells, and mitral cells of the olfactory bulb, all regions implicated in autism based on postmortem investigation (Bauman and Kemper, 1994).

Of the four known mechanisms of AS, deletion of 15q11–q13 on the chromosome of maternal origin leads to a more severely affected phenotype (Moncla et al., 1999; Lossie et al., 2001). This is likely to be due to deletion cases having only one copy of genes in the 15q11–q13 region that are not imprinted, including the GABA-A receptor subunit genes at 15q11–q13 (*GABRA5, GABRB3, GABRG3*). This may contribute to the phenotype of deletion cases, but not other forms of AS.

Most, if not all, cases of AS also meet the criteria for autistic disorder (Steffenburg et al., 1996). In one study of 100 consecutive cases of autistic disorder without a minimal IQ requirement for inclusion, one case had the deletion form of Angelman syndrome (Schroer et al., 1998).

PRADER-WILLI SYNDROME

PWS is one of the most extensively studied genetic disorders in terms of its behavioral phenotype. Physical features of PWS include neonatal hypotonia (often leading to feeding difficulties), hypogonadotropic hypogonadism, severe obesity, short stature, small hands and feet, almond-shaped eyes, and a narrow face with reduced bifrontal diameter. In addition to obesity, the behavioral phenotype includes noneating-related compulsive behaviors and stereotypies (State et al., 1999). PWS presents with cognitive impairment ranging from moderate mental retardation to borderline intellectual functioning (Descheemaeker et al., 2002).

Most cases (~70%) of PWS are due to de novo deletion of the same 15q11–q13 region as is typically deleted in AS (Ledbetter et al., 1981). Therefore, the remarkable difference in phenotype between AS and PWS is due to the parent of origin of the deletion. The majority of nondeletion cases of PWS (28% of all PWS) are due to maternal UPD, in which two otherwise normal copies of chromosome 15 are inherited only from the mother. In the case of maternal UPD, genes expressed only from the chromosome inherited from the father would not be expressed normally in PWS.

Dykens et al. (1999) has described an interesting contrast between PWS due to 15q11–q13 deletion compared to maternal UPD. Overall behavioral problems and cognitive impairment are greater in patients with 15q11–q13 deletion than in patients with maternal UPD, presumably due to the effect of deletion of one copy of nonimprinted genes in the region. In contrast, autism was only present in some of the cases with maternal UPD, in which overexpression of maternally expressed genes may be seen as well as decreased expression of paternally expressed genes (Dykens et al., 1999).

DUPLICATIONS OF 15Q11–Q13

Duplications of 15q11–q13 are the flip side of PWS and AS due to deletions. In fact, the mechanism of duplications is a reciprocal of the deletions. The boundaries of the duplications and deletions of the 15q11–q13 region are set by duplicons, relatively large regions of DNA that are similar at both ends of the region. The mechanism is similar to deletion and duplication syndromes at 17p11 and 22q11.

There are several classes of duplications of this region and adjacent regions. Duplications may be of maternal or paternal origin. They may be interstitial or lead to a supernumerary, marker chromosome. They may either contain the

15q11–q13 region, similar to typical deletions leading to PWS or AS, or they may only contain an expansion of repeats in a region proximal (closer to the centromere) than to the PWS/AS region. It is notable that the expansion of repeats proximal to the PWS/AS region is not thought to be related to autism or any other phenotype (Ritchie et al., 1998). Recently, further details about this region and probes useful for distinguishing between duplications of 15q11–q13 and expansion of this more proximal repeat have been published (Fantes et al., 2002).

Because the focus of this book is on autism, it may be helpful to note that the current data support that only interstitial or extrachromosomal duplications (or triplications) of the PWS/AS region of *maternal* origin greatly increase the risk for ASD. Supernumerary marker chromosomes of maternal origin appear to lead to the greatest risk for autism and more severe cognitive impairment and further increase in the risk for epilepsy, relative to interstitial deletions. In addition, although screening for these duplications has not yet become part of the routine assessment of children with ASD, recent surveys of the prevalence of this abnormality suggest that screening for 15q11–q13 duplications will be of higher yield than screening for fragile X mutations. G banded chromosomes will detect supernumerary marker chromosomes and may detect interstitial chromosome 15q11–q13 duplications, but fluorescence in situ hybridization (FISH) studies with probes from the PWS/AS region will typically be necessary to distinguish different classes of duplications, and microsatellite studies are necessary to determine the parent of origin of duplicated material. Methylation studies are also useful.

SUPERNUMERARY MARKER CHROMOSOMES INCLUDING 15Q11–Q13

Supernumerary chromosomes including the 15q11–q13 region have been described in patients with ASD in several reports (Baker et al., 1994; Bundey et al., 1994; Schroer et al., 1998; Wolpert et al., 2000; Borgatti et al., 2001). In all of the cases of supernumerary marker chromosomes containing the 15q11–q13 region with ASD, the marker chromosome has been of maternal origin. Epilepsy occurs at a high rate in these patients, and the level of mental retardation has typically ranged from severe to moderate. In one series of patients who were screened for this abnormality, 3 percent of patients with autism had this supernumerary chromosome. It is referred to as an *isodicentric chromosome 15* [idic (15)], or informally, as an *inverted duplicated chromosome 15* [inv dup (15)]. A support group, IDEAS, has been formed by parents, and an informative web site has been created at www.idic15.org. Supernumerary chromosomes that do not contain 15q11–q13 are thought to not increase risk for ASD, but often are associated with maternal UPD and therefore, may be related to risk for PWS.

Interstitial Duplications Including 15q11–q13

Interstitial duplications including 15q11–q13 appear to increase risk for ASD and/or seizures and/or developmental coordination disorders when of maternal origin compared to when they are of paternal origin (Cook et al., 1997; Bolton et al., 2001). The phenotype of patients with maternally derived interstitial duplications of 15q11–q13 is less severe in terms of risk for epilepsy and mental retardation than those with idic (15).

Possible Mechanisms of Increased Risk from Duplication of 15q11–q13

Given that there is an increased risk for ASD with duplications of maternal origin relative to paternal origin, it is likely that increased expression of gene(s) expressed in at least some brain regions at some times in development more from the chromosome of maternal origin than the chromosome of paternal origin increases the risk for ASD. The assumption that increased copy number in the duplications is associated with increased expression of at least one preferentially maternally expressed gene (*UBE3A*) has been demonstrated using RNA-FISH studies (Herzing et al., 2002). At least two genes in the 15q11–q13 region are known to be preferentially expressed from the maternal allele: *UBE3A* and *ATP10C*. Either or both of these genes, or possibly other genes in the region, may contribute to the increased risk for ASD in 15q11–q13 duplications of maternal origin.

Deletion of 15q22–q23

Smith and colleagues (2000) conducted FISH studies examining for a possible abnormality of 15q11–q13 in a patient with autism and serendipitously found that the polymyelocytic leukemia probe at 15q22–q23 used as a control for the 15q11–q13 region was deleted. They noted homologous DNA segments in 15q22–q23 and 15q11–q13.

Association and Linkage Studies of 15q11–q13

Although duplications of 15q11–q13 could have no implication for susceptibility for autism for the greater than 95 percent of patients with autism without 15q11–q13 duplications, the similarity of these patients to other patients with autism, particularly those with seizures or abnormal electroencephalograms has led to increased study of the 15q11–q13 region by association and linkage analy-

sis in patients without duplications. In fact, the first reported family demonstrating a difference between the phenotypes of paternally and maternally derived interstitial duplication of 15q11–q13 was found during an association study including microsatellite markers across 15q11–q13 (Cook et al., 1997).

Several studies have reported evidence of association of polymorphisms between *UBE3A* and *GABRB3* on 15q11–q13. The only 15q11–q13 marker in which two or more independent samples have shown nominal evidence of family-based association is a marker, *GABRB3* 155CA-2, in the 5′ end of the third intron of the GABA-A beta 3 subunit gene (Cook et al., 1998; Buxbaum et al., 2002). Several other studies have failed to find an association between this variant and autism (Maestrini et al., 1999; Salmon et al., 1999; Menold et al., 2001; Nurmi et al., 2001). One of these studies did show nominal evidence of association between a polymorphism 3′ of *GABRB3* (Menold et al., 2001). The GABA-A subunit genes on 15q11–q13 remain interesting candidates, given the finding of reduced hippocampal GABA-A receptor binding found in a neuropathologic study of autism (Blatt et al., 2001; see also Chapter 23).

Association was found with the marker D15S122, within *UBE3A,* in one study (Nurmi et al., 2001). Although it does not exclude other mechanisms (e.g., altered gene expression) related to *UBE3A* being involved, a small study did not find amino acid variants in subjects with autism (Veenstra-VanderWeele et al., 1999).

ATP10C is an interesting candidate, because it is maternally expressed in some tissues (Herzing et al., 2001; Meguro et al., 2001). Several amino acid variants were identified, but none were found to be significantly preferentially transmitted in one study (Kim et al., 2002). As in the studies of *GABRB3* and *UBE3A,* the small sample size used to detect association in a complex genetic disorder such as autism limits interpretation of the negative data at *ATP10C* and *UBE3A,* which remain excellent candidates, given their preferential maternal expression in some tissues.

Although several genome-wide scans have not found significant evidence of linkage by allele-sharing methods in the 15q11–q13 region, increased sharing by affected sibling pairs has been found consistently by one group (Bass et al., 2000). This group also reported increased recombination in the region in families with autism probands compared to control families.

Because the 15q11–q13 region is a candidate region on the basis of the phenotype of patients with 15q11–q13 duplications, it would make sense to study groups of subjects with autism that are more homogenous relative to the phenotype of maternally derived 15q11–q13 duplications. Additional features are present in many patients with these duplications, such as seizures and/or epileptiform electroencephalogram abnormalities, early hypotonia followed by clumsiness, and prominent restricted and repetitive behaviors. Selecting samples on

the basis of these additional features may increase the power to detect association and linkage to markers on 15q11–q13.

Future Directions

Future directions in the study of chromosome 15 and autism will include the study of more phenotypically homogenous groups of subjects, studies of mouse models of 15q11–q13 duplication, and fine mapping by linkage disequilibrium across 15q11–q13. In addition, larger-scale screening for duplications of 15q11–q13 in subjects with and without autism will be of interest. Further exploration of the phenotype of various classes of duplications of 15q11–q13, such as by neuroimaging, neuropsychological assessment, and study of a larger number of families will assist in determining the range of phenotypes associated with these duplications and will lead to a better understanding of the relationship between genotype and phenotype. Expression microarray studies may assist in elucidation of the effect of 15q11–q13 duplication on the expression of genes from other genomic regions.

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Chromosome 7

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Genome Screen Findings

Initial evidence that the long arm of chromosome 7 (7q) may contain a gene or genes relevant to autism came from the first genetic linkage study of autism, published by the International Molecular Genetics Study of Autism Consortium in 1998. Using markers spanning the genome at approximately 10-centiMorgan (cM) intervals, this group reported evidence for *suggestive linkage* (logarithm of the odds [LOD]: 2.53 in 87 affected sibling pair families) in a fairly large interval around 7q31 (Figure 18.1). A follow-up genome screen by the International Molecular Genetics Study of Autism Consortium (2001) using 69 additional families improved the evidence for linkage to 7q (LOD of 3.2). The Collaborative Linkage Study of Autism (1999) also found evidence for *suggestive linkage* (LOD: 2.2) to the same region of 7q, in a genome screen using 75 affected sibling pair families. In addition, researchers at Duke University (Shao et al., 2002) identified the 7q interval as promising (although the LOD of 1.66 did not meet the Lander and Kruglyak, 1995, criteria for *suggestive linkage*) in a two-stage genome screen with 99 multiplex families.

The remaining four published genome screens have not yielded evidence for *suggestive linkage* to 7q, and some researchers have expressed the opinion that true evidence for linkage in complex diseases will only be possible with a significant increase in the number of families and a study sample limited to a specific ethnic group (Altmüller et al., 2001). Nevertheless, authors of a meta-analysis (Badner and Gershon, 2002:67) that included the International Molecular Genetics Study of Autism Consortium (1998), the Collaborative Linkage Study of Autism (1999), Paris (Philippe et al., 1999), and Stanford University (Risch et al., 1999) studies concluded that "evidence of significant and replicated linkage has been found to 7q despite the fact that none of the individual studies exceeded

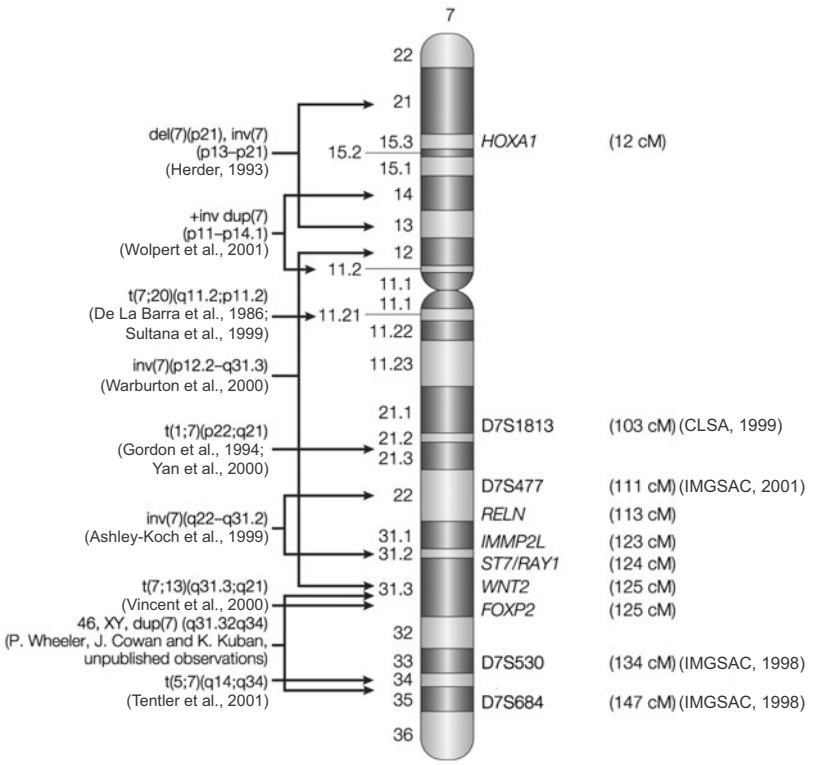

FIGURE 18.1. Regions of chromosome 7 that are implicated in autism. On the left are examples of reported cytogenetic abnormalities in autism probands. On the right are markers for which genome screens have obtained suggestive or significant LOD scores, as well as candidate genes under study. cM, centiMorgan; del, deletion; dup, duplication; *FOXP2,* forkhead box P2; *HOXA1,* homeobox A1; *IMMP2L,* inner mitochondrial membrane peptidase 2-like; inv, inversion; *RAY1/ST7,* suppression of tumorigenicity 7; *RELN,* Reelin; *WNT2,* wingless-type MMTV integration site family member 2. *Source:* Folstein and Rosen-Sheidley (2001). Reprinted by permission from *Nature Reviews Genetics,* copyright 2001 Macmillan Magazines, Ltd.

Lander and Kruglyak criteria for *significant linkage."* It would be perhaps even more illustrative to undertake a meta-analysis of all seven published genome screens for confirmation of 7q as a likely susceptibility locus. Regardless, and despite the converging evidence from work to date, the interval on 7q is very large and contains far too many genes to analyze individually, given the current state of technology.

Narrowing the Interval

The next step, currently under way, has been to narrow the interval sufficiently to decrease the possible candidate genes to a more manageable number. This is being approached in several ways, and when all these approaches provide converging evidence, the arduous work of sequencing individual genes can become more focused. One approach is to find affected individuals who have chromosome anomalies, such as duplications or deletions, involving the candidate region (Figure 18.2). In some instances, the breakpoint of a chromosome anomaly contains a gene that, when disrupted, may confer risk for the disorder. Several such chromosome anomalies have been reported on chromosome 7 (see Figure 18.1), and the genes at the breakpoints are under study.

A second approach to narrowing the interval is to add additional markers more closely spaced than 10 cM (referred to as *fine mapping*), but this only sometimes results in a narrower region. Another approach is to choose the subset of families that have a positive linkage signal in the overall region and define the smaller interval that is shared by the majority of the sibling pairs. This approach is most effective if combined with fine mapping. Finally, it is sometimes possible to find naturally occurring or genetically engineered mouse mutants that have a phenotype, either behavioral or neuroanatomical, that bears some resemblance to the disease under study. For chromosome 7q31–32, these approaches have indeed narrowed the interval and thus the possible number of candidate genes.

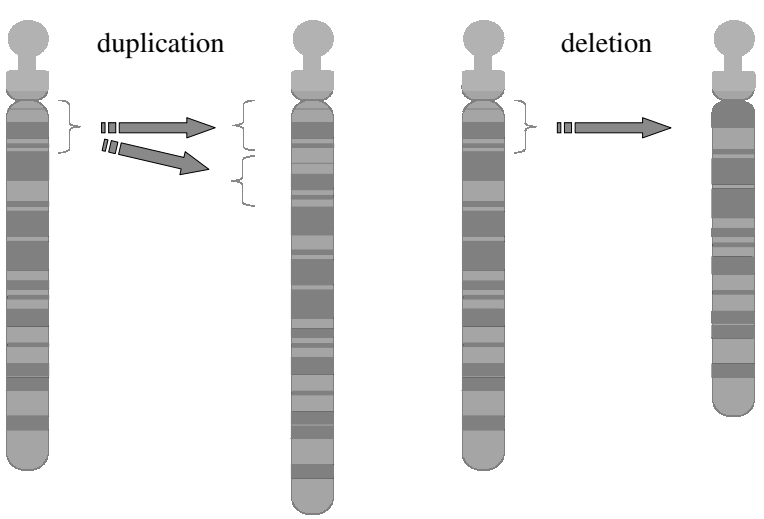

FIGURE 18.2. Examples of structural chromosome abnormalities.

Candidate Genes

RAY1/ST7

The candidates examined thus far have been primarily at 120–130 cM within 7q31, which, based on the preponderance of evidence, is the most likely interval to harbor an autism-related gene. Vincent et al. (2000) identified an autistic individual with a translocation involving chromosomes 7q and 13q, and determined that a novel gene was interrupted by the translocation breakpoint. Mutation screening of the coding regions of this gene, *RAY1,* did not initially yield evidence for involvement in the etiology of autism. Zenklusen et al. (2001) reported *RAY1* to be a tumor suppressor gene, *ST7,* but subsequent studies have yielded conflicting results (Thomas et al., 2001; Hughes et al., 2001). However, there are new findings emerging from this locus. Vincent et al. (2002) report on a more detailed study of this region, which has an extremely complex structure and appears to consist of a multigene system with multiple isoforms and alternative splicing. Sequence variants identified in several unrelated autism probands may possibly be related to the autism phenotype, but the significance of the findings are unclear at this time (Vincent et al., 2002).

WNT2

Wassink et al. (2001) examined *WNT2* (*wingless-type MMTV integration site family member*), the gene adjacent to *ST7* on 7q31. It is one of more than a dozen *WNT* genes that are expressed during development in several different tissues, and in particular, those of the nervous system (Cadigan and Nusse, 1997). Furthermore, transmission of the WNT signal is dependent on the disheveled (DVL) protein family, and Lijam et al. (1997) described an unusual phenotype in *Dvl1* knockout mice. The phenotype was notable for reduced social interaction, including observed lack of huddling during sleep, the absence of grooming of cage mates, and diminished mothering behaviors. Wassink and colleagues identified two unrelated sibling pair families, among 75 examined, in which both affected siblings had coding sequence mutations. Each of the two families had a different mutation, but in both families, only the affected siblings and one of the two parents were found to have *WNT2* mutations. Neither the unaffected siblings nor any of the 160 control subjects were found to have sequence variations in *WNT2.* Significantly, the mutations found in both families changed the amino acid sequence and charge of the protein, and they occurred in coding regions of the gene that had been conserved across species.

Wassink et al. (2001) also identified an association between autism and a more common variation in the gene. The variation, referred to as a *single nucleotide*

polymorphism (SNP), occurs in a noncoding region of the *WNT2* gene, and was found more frequently than expected among the group of 75 sibling pair families and an additional 45 trio families. Within the group of 120 families studied, the association with the SNP was found to originate almost entirely from the subset of families identified as having severe language abnormalities. These findings suggest that, although occasionally severe mutations in *WNT2* may be sufficient to cause autism, variations in the gene may act more commonly as susceptibility alleles.

Of note, McCoy et al. (2002) screened 135 trio families and 82 multiplex families for the *WNT2* mutations identified by Wassink et al. (2001) and did not identify any mutations in the coding region of the *WNT2* gene. McCoy and colleagues also tested the entire group of 217 families for association with two different SNPs in noncoding regions of *WNT2,* but found no evidence for association, even when the families were grouped by the presence or absence of language impairment. The authors note that differences in the study population may account for the inability to detect an association, but more work will be needed to determine the significance of *WNT2* as a susceptibility gene.

RELN

Located on chromosome 7q22, the *RELN* gene encodes Reelin, a signaling protein that plays a crucial role in the development of the mammalian brain. Studied extensively in mice, Reelin has been shown to affect the migration of cortical neurons (Goffinet, 1984, 1990; D'Arcangelo et al., 1995; Hoffarth et al., 1995; Ogawa et al., 1995; Miyata et al., 1996; Del Río et al., 1997). Naturally occurring mice lacking Reelin, known as *Reeler* mice, have been shown to have developmental brain alterations (Goffinet, 1984, 1990; Del Río et al., 1997) similar to those observed in the brains of individuals with autism (Bauman, 1991; Rodier et al., 1996; Courchesne, 1997; Bailey et al., 1998). These include hypoplasia of the cerebellum, brainstem abnormalities, and defects in neuronal migration (Lambert de Rouvroit and Goffinet, 1998). Hong et al. (2000) studied two unrelated families with an autosomal recessive form of lissencephaly and identified two different *RELN* mutations that led to low or undetectable amounts of Reelin protein in the affected individuals. Persico et al. (2001) reported on an association between autism and a polymorphism of a noncoding region of *RELN.* The polymorphism consisted of a repetitive DNA sequence (such as those identified in fragile X syndrome and Huntington disease); autistic patients were more frequently identified as having longer repeats, whereas unaffected siblings/parents/controls more often were found to have shorter repeats. Thus, it is possi-

ble that polymorphisms or as yet unidentified mutations in *RELN* may confer susceptibility for autism. However, additional work will be needed to confirm the preliminary findings.

FOXP2

Formerly referred to as *SPCH1, FOXP2* is a gene related to a developmental language disorder in the 7q31 region (Lai et al., 2001). Lai and colleagues cloned *FOXP2* and identified a point mutation encoding a transcription factor in affected members of the KE family (first described by Hurst et al. in 1990), who are affected by a severe speech and language disorder. Lai also demonstrated that *FOXP2* was disrupted by a chromosome translocation breakpoint in a patient unrelated to the KE family. Thus far, no mutations of *FOXP2* have been identified in families with autism (Newbury et al., 2002; Wassink et al., 2002), but it is possible that autism and specific language impairment (SLI) may share a gene in this region of 7q (Folstein and Mankoski, 2000). This possibility is based on two pieces of evidence. First, many individuals with autism, as well as some of their family members, have abnormalities of structural language that resemble those seen in SLI. Second, in the Collaborative Linkage Study of Autism dataset, nearly all the linkage signal on chromosome 7q was attributable to the families in which the probands have severely delayed onset of phrase speech (Collaborative Linkage Study of Autism, 2001). This finding has been replicated in a different dataset, but the subsetting was based on onset of first single words rather than phrase speech (Alarcon et al., 2002).

IMMP2L

Petek and colleagues (2001) identified a patient with Tourette syndrome and a de novo inverted duplication of 7q [46,XY,dup(7)(q22.1–q31.1)]. Analysis of the breakpoints revealed that the distal breakpoint occurred in the same region of 7q in which a familial form of Tourette had been reported in association with a chromosome 7 translocation [t(7;18)(q22–q31;q22.3)]. Sequence analysis of the 7q31 breakpoint in the patient with the inverted duplication revealed a disruption of a newly identified gene referred to as *inner mitochondrial membrane peptidase 2–like* (*IMMP2L*). Given the increased rate of Tourette symptoms in patients with autism and the additional evidence for involvement of loci on 7q, the authors speculate that the *IMMP2L* findings could be relevant to the search for autism related genes.

Summary

The chromosome 7q region continues to be the focus of much interest to those searching for autism susceptibility genes. The combined evidence from genome screens, naturally occurring chromosome anomalies in patients with autism, and findings from association studies to date indicates that this region may contain one or several of the genes related to the autism phenotype. Note that, once identified, the alleles will most likely be found to be normally distributed in the population as a whole. Only when they are inherited with other susceptibility alleles, and specific environmental insults occur as well, would they lead to a phenotype noticeable enough to come to clinical attention. Likewise, the environmental risk factors with which they are hypothesized to interact are probably also commonly experienced by many individuals in the population without untoward effects. Due to this complexity, it is not likely that predictive testing will be particularly effective. Nevertheless, identification of susceptibility alleles will lead to a better understanding of the underlying mechanisms, and, hopefully, a new generation of treatments for individuals with autism.

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19

Fragile X Syndrome

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Fragile X syndrome (FXS) is the most common inherited cause of mental retardation known and has a strong association with autism. Approximately 2–6 percent of males with autism have FXS, and 15–33 percent of children with FXS have autism (Bailey et al., 2001b; Rogers et al., 2001; Hagerman, 2002). FXS represents a distinct subgroup of autism, and the study of the behavioral phenotype, neurobiology, and molecular genetics of FXS could lead to new insights regarding pathophysiology that may be applicable to other causes of autism. FXS is a model of how a single-gene disorder can affect the expression of multiple genes that may be held in common with other etiologies of autism.

FXS is caused by a trinucleotide repeat expansion at Xq27.3, which is at the bottom end of the X chromosome. Individuals in the general population have between 5 and 50 CGG repeats, with an average number of 29. Individuals who are carriers for FXS have the premutation, which is 55–200 CGG repeats. Those with the premutation are usually, but not always, intellectually normal. Individuals with the full mutation, more than 230 CGG repeats, have a full mutation that is usually fully methylated, meaning that methyl (CH_3) groups are added to the backbone of the DNA. This typically shuts down transcription of messenger RNA (mRNA) with a subsequent lack of FMR1 protein (FMRP) production (Bardoni and Mandel, 2002). It is the lack or deficiency of FMRP that causes FXS. Individuals with a mild deficiency of FMRP are typically high functioning, with a normal or near normal IQ, although learning disabilities and behavioral problems are common. Individuals with an absence of FMRP are usually mentally retarded, ranging from mild to severe. Females are less affected with the full mutation because they have a second X chromosome, which is typically producing some level of FMRP, depending on the X-inactivation status of individual cells. Approximately 70 percent of females with the full mutation will have a borderline or lower IQ in the mentally retarded range (de Vries et al., 1996).

The prevalence of FXS associated with mental retardation is approximately 1 in 4,000 (Turner et al., 1996). However, this prevalence figure does not include individuals who are higher functioning, with normal or borderline IQ, who may present with learning disabilities or emotional and behavioral problems. If these individuals are included, the prevalence figures may be closer to 1 in 3,000 males in the general population. Prevalence studies have not been carried out in females, and a significant number of females do not present with mental retardation.

The prevalence of the premutation is significantly higher than that of the full mutation. Studies by Rousseau et al. (1995, 1996) found that 1 in 259 women and 1 in 760 males in the general population have more than 54 CGG repeats. If a female has more than 90 CGG repeats, her risk of having this allele expand to the full mutation when it is passed to the next generation is virtually 100 percent (Gane and Cronister, 2002). The premutation will expand to a full mutation only when it is passed by a female carrier to the next generation. Male premutation carriers will pass the premutation to all of their daughters but to none of their sons. For a review of prevalence studies, see Sherman (2002).

Neurobiology

FMRP is a RNA binding protein that regulates the translation of mRNA (Bardoni and Mandel, 2002). FMRP binds to approximately 4 percent of neuronal messages, and recent microarray studies have been helpful in identifying these messages (Brown et al., 2001; Darnell et al., 2001). When FMRP is absent, approximately 400 mRNAs are dysregulated; it is thought that this translational dysregulation is the cause of FXS. Many of the messages that are dysregulated in the absence of FMRP are associated with synaptic and dendritic structure. FMRP is thought to be critical in early development of maturing dendritic spines and subsequently to be involved in paring down dendritic connections, which is a normal process in brain maturation (Irwin et al., 2002). Human autopsy studies of patients with FXS demonstrate an enhanced number of dendritic connections that are also immature in structure (Irwin et al., 2000).

Physical and Behavioral Features

The typical physical features of FXS in adults include a long face, prominent ears, and macroorchidism (large testicles). These features are seen in approximately 60–80 percent of males with the full, completely methylated mutation (Hagerman, 2002). However, young children typically do not demonstrate macroorchidism, and at least a third do not demonstrate prominent ears or a long face (Figure 19.1). The physical features are thought to be secondary to a

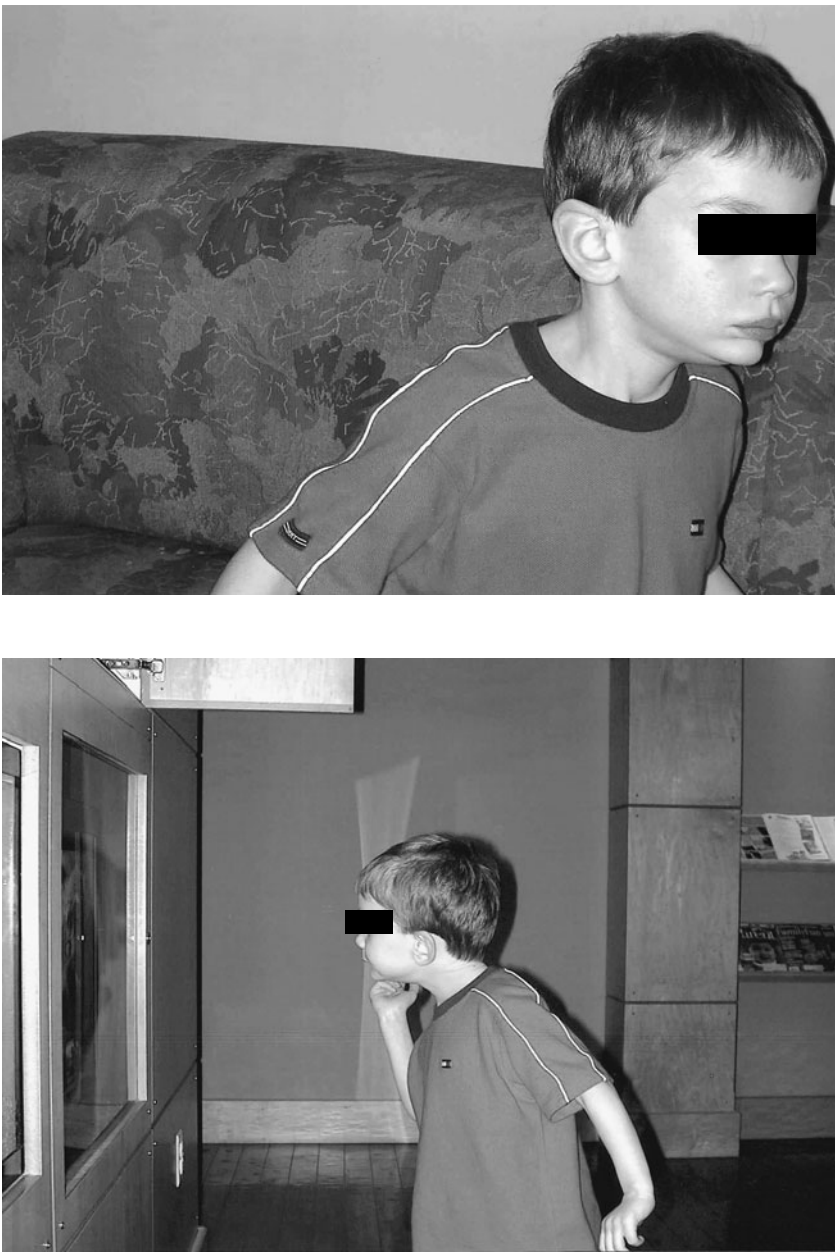

FIGURE 19.1. A 6-year-old boy with fragile X syndrome and autism. Note the normal facial features and hand mannerisms.

connective tissue dysplasia, and there are abnormal elastin fibers in the skin of individuals with FXS (Waldstein et al., 1987). Other features related to the connective tissue abnormality include flat feet, hyperextensible finger joints, double-jointed thumbs, and soft, smooth skin. Approximately 50 percent of adults with FXS will have mitral valve prolapse, but this is less common in childhood. Both mitral valve prolapse and mild dilation at the base of the aorta are caused by abnormal elastin fibers.

Approximately 20 percent of individuals with FXS have seizures that are usually well controlled in early childhood, and can include grand mal, petit mal, and absence episodes (Musumeci et al., 2000). Strabismus is also commonly seen in childhood, with 8–30 percent of affected children demonstrating this feature (King et al., 1995; Hatton et al., 1998).

Men with FXS are fertile, but in individuals with the full mutation, the sperm only contains the premutation. Therefore, men with FXS will pass only the premutation to their daughters in the next generation. Sons will receive the Y chromosome and will be unaffected by FXS.

Approximately 80 percent of males with FXS exhibit behaviors consistent with attention deficit hyperactivity disorder (ADHD). Significant problems with anxiety occur in the majority of males and females with FXS. Tantrums, mood instability, and panic attacks are also relatively common. Anxiety appears to be exacerbated by the patient's overreactivity to sensory stimuli. This has been documented in psychophysiologic studies (Belser and Sudhalter, 1995). For instance, individuals with FXS have an enhanced sweat response to all sensory stimuli, including visual, tactile, auditory, olfactory, and vestibular. In electrodermal studies, the amplitude of the sympathetic response is dramatically enhanced, and there is a lack of normal habituation (Miller et al., 1999). Parasympathetic tone has also been documented to be decreased in heart rate studies of children with FXS (Boccia and Roberts, 2000). There is also a lack of appropriate modulation of autonomic influences on cardiovascular parameters in active versus passive tasks in patients with FXS compared to controls (Roberts et al., 2001). Patients with autism and FXS together have even further dysregulation of sympathetic and vagal tone compared to those children with FXS alone (Roberts et al., 2001). When individuals with FXS are hyperaroused by environmental stimuli, their behaviors include hand flapping, hand biting, or poor eye contact and perseverative behaviors that are often labeled "autistic-like," but that are also associated with the clinical perception of increased anxiety.

For females with FXS, anxiety is often a presenting feature (Freund et al., 1993; Lachiewicz and Dawson, 1994), because other behavioral problems, such as ADHD or aggression, are less severe than in males. The occasional presence of

selective mutism in girls with FXS is also related to their anxiety (Hagerman et al., 1999). Hessl et al. (2001) reported a significant negative association between FMRP levels and internalizing behaviors on the Child Behavior Checklist, particularly socially withdrawn and anxious/depressed behaviors in girls with FXS. The association is independent of the effect of FMRP on IQ. The Hessl et al. report represents one of the first on FMRP's association with behavior measures.

Studies of the hypothalamic-pituitary-adrenal (HPA) axis have demonstrated enhanced levels of cortisol production, particularly when under stress, in children with FXS compared to their normal sibling controls (Hessl et al., 2002). Hessl and colleagues suggested either a direct effect of the FMRP deficit on the HPA axis, as was also suggested by Loesch et al. (1995) because of growth abnormalities in FXS, or a secondary effect, such that the hyperarousal and enhanced reactivity to environmental stimuli may lead to enhanced stress and subsequent elevated cortisol release. The long-term sequelae of elevated cortisol may influence the neurobehavioral phenotype in FXS and may also affect the size and function of the hippocampus, which can experience cell death with chronic stress and elevated cortisol levels (Sapolsky, 2000).

Approximately 10 percent of children with FXS may be nonverbal, but for those who do talk, rapid and cluttered speech is typically observed. Their speech also has significant dysfluencies, pat phrases, and the use of highly associated words, such as answering "trunk" for a question about elephants, even though it may be the wrong response (Sudhalter et al., 1990; Scharfenaker et al., 2002).

Cognitive Features

MALES

Males are more significantly affected than females with FXS, because they have only one X chromosome. Cognitive abilities are typically in the mild to moderately retarded range. The average IQ for a man with a fully methylated full mutation is 41, whereas younger children may present with a borderline or only mildly retarded IQ. The IQ correlates with the FMRP level in both males and females (Tassone et al., 1999). Therefore, males who have an unmethylated full mutation or a mosaic pattern—meaning that some cells have a premutation and some have a full mutation—will often have an IQ of 70 or higher, because they are producing more FMRP than those with a full mutation that is fully methylated.

There is an IQ decline with age that occurs in the majority of males who have little or no FMRP (Wright Talamante et al., 1996). Bailey et al. (1998a) carried out

longitudinal studies in 46 boys with FXS diagnosed early in childhood and demonstrated developmental growth at only half the rate seen in their normal peers, with a relative weakness in communication and cognitive scales compared to motor and adaptive scales.

INVOLVEMENT IN THE PREMUTATION

It is apparent that FMRP is important to neuronal cells throughout their lifetime. Recently, a tremor/ataxia syndrome was described in a subgroup of older males with the premutation who were normal cognitively during their lives but had low normal or mildly deficient FMRP levels (Hagerman et al., 2001; Jacquemont et al., 2003). This syndrome can begin with an intention tremor, which interferes with handwriting and may gradually worsen and affect other activities of daily living, such as eating and dressing. Ataxia may also develop, with frequent falling. Magnetic resonance imaging (MRI) studies show generalized brain atrophy particularly involving the cerebellum. A study by Brunberg et al. (2002) demonstrated a characteristic radiologic sign of bilateral hyperintensities of the middle cerebellar pedunicles on T_2 images in premutation males who have the tremor/ataxia syndrome.

The cause of this syndrome is not known, but it may be related to the dysregulation of the *FMR1* gene that occurs in the premutation. Individuals with the premutation have demonstrated an elevation of the FMR1 mRNA levels, with either mildly decreased or low normal FMRP levels (Tassone et al., 2000a, 2000b, 2000c). Those with the premutation have a block in the translation of the mRNA (which has the CGG repeats on it) into FMRP and presumably mRNA levels increase to keep FMRP levels normal or near normal. Neuropathologic studies have been carried out in four males who have died from this tremor/ataxia syndrome, and all four have demonstrated eosinophilic intranuclear inclusions in 5–30 percent of neurons and 20–40 percent of astroglia cells throughout the brain (Greco et al., 2002). Although Purkinje cells in the cerebellum have not demonstrated these inclusions, there is evidence of degeneration in the cerebellum that leads to its atrophy (Greco et al., 2002). The inclusions may be related to excess mRNA, gene dysregulation, or problems in the ubiquination or breakdown of proteins in the neurons. Further studies are in progress to detect whether a second gene effect defines the subgroup of premutation males who develop this syndrome.

Autism can also be seen occasionally in children with the premutation (Tassone et al., 2000c; Aziz et al., 2003). This may be related to lower protein levels, which sometimes occur with the permutation, or to toxic effects of the elevated mRNA levels (Tassone et al., 2000b, 2000c).

FEMALES

Approximately 70 percent of females with the full mutation have an IQ of less than 85 (de Vries et al., 1996). Most are in the borderline range, but a gradual IQ decline can lead to mild mental retardation in adulthood. An occasional female affected by FXS will have an IQ in the moderate or severe range of mental retardation (Bennetto and Pennington, 2002). More severely affected females may also have autism, as described below.

The level of cognitive involvement in females with the full mutation correlates with their X-inactivation ratio; that is, the percentage of cells that have the normal X as the active X chromosome. This correlates closely with overall FMRP levels measured in the blood (Tassone et al., 1999). Because there may be significant variability between the X-inactivation ratio or FMRP level in blood versus brain tissue, the correlations between these genetic variables and IQ in females are lower than is seen in males. In females, the molecular variables predict approximately 30 percent of the variability in IQ (Tassone et al., 1999).

Approximately 30 percent of females with the full mutation have an IQ in the normal range or above 85 (de Vries et al., 1996). However, executive function deficits are seen in the majority of females with a normal IQ (Bennetto et al., 2001). Furthermore, the degree of executive function deficit correlates with the degree of schizotypal features, including odd communication patterns and unusual mannerisms. Cognitive deficits in females also include visual-spacial deficits, which are most severe in the visual-constructive areas (Cornish et al., 1998).

NEUROIMAGING AND ELECTROPHYSIOLOGIC STUDIES

An important finding on MRI studies is a smaller posterior cerebellar vermis in individuals affected by FXS compared to controls. A study by Mostofsky et al. (1998) found that the size of the posterior cerebellar vermis was a significant predictor of performance on most of the cognitive measures, including verbal, performance, and full-scale IQ, the Rey-Osterreith Complex Figure Test, and the categories achieved on the Wisconsin Card Sorting Test. An additional study by Mazzocco et al. (1997) in females with FXS demonstrated that the size of the posterior cerebellar vermis correlated inversely with measures of stereotypic/restricted behavior, communication dysfunction, and autistic items on a neuropsychiatric developmental interview.

Other findings on MRI in FXS include an overall large brain, enlarged ventricles, and enlarged caudate and hippocampus compared to controls (Reiss et al., 1994; Shapiro et al., 1995; Eliez et al., 2001). There also appears to be a volumet-

ric decrease with age of the cortical gray matter, particularly caudate and thalamic gray matter, and an increase in the volume of the central nervous system with age (Reiss et al., 1994; Eliez et al., 2001). These changes with age may relate to the IQ decline frequently seen in FXS.

Functional MRI (fMRI) studies have also demonstrated consistent findings in girls with FXS. Math and working memory tasks have shown a lack of intensity of activation in multiple areas (Kwon et al., 2001; Rivera et al., 2002). There is a significant correlation between level of FMRP and brain activation in frontal and parietal regions. Therefore, more involvement from FXS leads to a restriction of the neural net of activation. Treatment interventions should focus on enhancing the neural net, so that more pertinent brain regions are recruited for problem solving.

Association between FXS and Autism

Brown et al. (1982) were the first to point out an association between autism and FXS. They evaluated 27 males with FXS and found that five (18.5%) were diagnosed with autism. This report was confirmed by several others (Levitas et al., 1983; Varley et al., 1985) and generated increased screening of males with autism for FXS. Some studies found no positives (Goldfine et al., 1985), whereas others showed a significant prevalence, with a high of 15.7 percent of males with autism having FXS in Sweden (Blomquist et al., 1985). In summary reports, approximately 6.5 percent of males with autism tested positive for FXS using cytogenetic studies (Brown et al., 1986).

With the advent of DNA testing after the *FMR1* gene was discovered in 1991, a more exact way to diagnose the CGG expansion became available. A study by Li et al. (1993) used both cytogenetic and DNA studies to screen 104 autistic children in Taiwan (84 boys and 20 girls). Eight (7.7%) were positive for FXS by DNA testing, but the cytogenetic expression was only 1 or 2 percent in five of the eight positive patients. This demonstrates that DNA testing is more precise in identifying individuals affected by FXS and will, on occasion, be positive when the cytogenetic expression is very low or even negative.

Another factor that has confused the association between FXS and autism is the changing diagnostic tools and diagnostic criteria for autism over the years. Previous studies of children and adults with FXS have demonstrated that 15–25 percent have autism, but part of this variability relates to different tools for making the diagnosis of autism (Hagerman et al., 1986; Reiss and Freund, 1992; Baumgardner et al., 1995; Cohen, 1995; Turk and Graham, 1997; Bailey et al., 1998b).

Bailey et al. (1998b) directly compared 57 young boys with FXS to a referral group of 391 individuals with autism on the Childhood Autism Rating Scale (CARS). They found that 25 percent met the CARS criteria for autism and had a very similar profile to the children with autism. They also found that individuals with a lower developmental level were more likely to be diagnosed with autism, as were all of the nonverbal children in their group with FXS. In a direct comparison between 31 children with FXS and autism compared to gender- and aged-matched children with autism without FXS, boys with autism alone had a more variable profile on the Battelle Developmental Inventory than did children with both FXS and autism (Bailey et al., 2000). Cohen and colleagues (1989, 1991) also carried out studies of eye gaze in children with autism compared to children with FXS. They found that children with autism tended to avoid direct eye gaze whether people were looking at them or not, whereas children with FXS were more avoidant of direct eye contact only when someone looked directly at them. This suggests a more reactive avoidance of eye contact in FXS instead of an indifference to eye contact in those with autism without FXS.

Rogers et al. (2001) directly compared 27 children with idiopathic autism, 24 children with FXS, and 23 children with other developmental delays who did not have autism and did not have FXS. She and her colleagues used state-of-the-art autism measures, including the Autism Diagnostic Interview (ADI-R) and the Autism Diagnostic Observation Scale (ADOS-G), as well as measures of development and adaptive behavior. Two distinct groups emerged from those subjects who had FXS. One subgroup of these children did not meet criteria for a diagnosis of autism, whereas 33 percent of the total FXS group met all criteria for autism on the ADOS-G, ADI-R, the DSM-IV, and the clinician's rating. Their profiles on the autism instruments were virtually identical to those of the group with idiopathic autism, whereas children in the subgroup of FXS who did not have autism were indistinguishable from those with developmental delay without FXS. The group with both FXS and autism scored lower developmentally on the Mullen Scales of Early Learning than did the group with idiopathic autism or that with FXS alone. This was similar to the findings of Bailey et al. (2000). Furthermore, a study by Bailey et al. (2001a) did not find a correlation between the presence or absence of autism and the level of FMRP. This additional information suggested that those with both autism and FXS together may have a double hit, which did not relate to their severity of FXS or the deficit of FMRP. The possibility of a second genetic hit causing autism in those with FXS was also suggested by Feinstein and Reiss (1998).

A recent study by Rogers et al. (2002) of preschoolers with FXS, both with and without autism, compared to preschoolers with autism and developmentally

delayed controls found that the imitation abilities of those with FXS correlated inversely with the pervasiveness of autism symptoms, as measured by the ADOS-G algorithm score. Those children with both FXS and autism had an imitation deficit, and the pattern of imitation problems showed difficulties that were most remarkable for oral motor and manual imitation—a different pattern compared to children with FXS without autism.

Language deficits may be another area that can differentiate children with FXS and autism from those without autism. Philofsky et al. (2004) recently carried out a study comparing children on the Mullen Scales of Early Learning who had FXS without autism, FXS with autism, autism without FXS, and controls with Down syndrome and typically developing children matched on nonverbal developmental age. They found that children with FXS without autism had the highest receptive language age, and those with FXS and autism were the most impaired on both receptive and expressive skills compared to all other groups. Thus, the severity of the receptive language deficit can also influence the presence of autism in those with FXS.

The work of Hessl et al. (2001) also brought into focus the influence of the environment on the presence of autism with FXS. They conducted an in-home evaluation of 120 children (80 boys and 40 girls) with the full mutation and their siblings. They evaluated autism with the Autism Behavior Checklist and the home environment with the Home Observation Measurement of the Environment scale. In boys with FXS, autistic behavior increased linearly as the quality of the home environment decreased. Maternal report of more effective educational and therapeutic services at school was associated with fewer autistic symptoms. Also of importance was the finding that children whose fathers did not participate in the study had significantly more autistic symptoms than did the children of participating fathers. Although having an organized home and a cooperative and participating father are features of the environment that predict an absence of autism in FXS, they may also have a genetic component. Mothers who have difficulty maintaining an organized and supportive home, and absent fathers may have more genetically based psychopathology or even the extended autism family phenotype. The bulk of the evidence presently points to multiple factors, including both genetic and environmental, which correlate with the presence of autism in FXS.

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20

Autism and Tuberous Sclerosis Complex

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Autism is a severe neurobehavioral disorder that often occurs in the presence of other medical conditions, such as seizure disorders, chromosomal anomalies, and specific genetic syndromes (Smalley et al., 1998). Tuberous sclerosis complex (TSC) is a genetic syndrome associated with autism. The mechanism of the association between TSC and autism is as yet unclear. This chapter describes the clinical phenotype and etiology of TSC, the extent of association of TSC and autism, and the molecular and neurobiologic data addressing possible underlying mechanisms of the pathogenesis of autism in TSC.

Tuberous Sclerosis Complex

TSC is a tumor-suppressor gene syndrome whose incidence is approximately one in 10,000 births (O'Callaghan et al., 1998). The manifestations of TSC include seizures, mental retardation, and tumors of the brain, retina, kidney, heart, and skin (Gomez et al., 1999). The revised diagnostic criteria for TSC (Roach et al., 1998) are shown in Table 20.1. The most frequent tumors in TSC include cerebral cortical tubers, subependymal giant cell astrocytomas, facial angiofibromas, cardiac rhabdomyomas, pulmonary lymphangiomyomatosis, and renal angiomyolipomas.

As indicated in Table 20.1, the physical features of TSC vary quite dramatically, in much the same way that autism shows a high degree of variability in behavioral domains, such as social, language, and repetitive rituals. However, in contrast to autism, with its likely complex genetic etiology, TSC is inherited in an autosomal dominant fashion with high penetrance.

GENETICS

Germline mutations in either of two genes, *TSC1* and *TSC2,* cause TSC. The *TSC1* gene, located on chromosome 9q34, and the *TSC2* gene, located on 16p13, were

TABLE 20.1. Revised Diagnostic Criteria for Tuberous Sclerosis Complex

Major features	Minor features
Facial angiofibromas or forehead plaque	Multiple, randomly distributed pits in dental enamel
Nontraumatic ungual or periungual fibroma	Hamartomatous rectal polyps[c]
Hypomelanotic macules (three or more)	Bone cysts[d]
Shagreen patch (connective tissue nevus)	Cerebral white matter radial migration lines[a,d,e]
Multiple retinal nodular hamartomas	Gingival fibromas
Cortical tuber[a]	Nonrenal hamartoma[c]
Subependymal nodule	Retinal achromic patch
Subependymal giant cell astrocytoma	"Confetti" skin lesions
Cardiac rhabdomyoma, single or multiple	Multiple renal cysts[c]
Lymphangiomyomatosis[b]	
Renal angiomyolipoma[b]	

Source: Roach et al. (1998).

Note: Definite TSC: either two major features, or one major feature plus two minor features; Probable TSC: one major plus one minor feature; Possible TSC: either one major feature or two or more minor features.

[a] When cerebral cortical dysplasia and cerebral white matter migration tracts occur together, they should be counted as one rather than two features of tuberous sclerosis.

[b] When both lymphangiomyomatosis and renal angiomyolipomas are present, other features of tuberous sclerosis should be present before a definite diagnosis is assigned.

[c] Histologic confirmation is suggested.

[d] Radiographic confirmation is sufficient.

[e] One panel member (Manuel R. Gomez) felt strongly that three or more radial migration lines should constitute a major sign.

identified by positional cloning (Consortium TECTS, 1993; van Slegtenhorst et al., 1997). Although the *TSC1* gene was localized first (Connor et. al., 1987), the *TSC2* gene was cloned first in 1993. The *TSC2* gene is composed of 41 exons spanning 40 kb of chromosome 16p13. The transcript is 5.4 kb in length (European Chromosome 16 Tuberous Sclerosis Consortium, 1993) and encodes tuberin, a 200-kD protein (1,784 amino acids) with a domain homologous to the Rap1 GTPase activating protein (GAP). The *TSC1* gene, cloned in 1997, has 21 coding exons, spans 20 kb of chromosome 9q34, and codes for a transcript 3.4 kb in length. The resulting protein, hamartin, is a 140-kD protein (1,164 amino acids) with no homology to tuberin (van Slegtenhorst et al., 1997).

Multiple lines of evidence indicate that *TSC1* and *TSC2* are tumor suppressor genes fitting the Knudson (1971) "two-hit" model. A tumor-suppressor gene is one in which the gene product inhibits a cell's proliferation or activity; by analogy, being the "brake" on a specific activity. As a tumor suppressor gene, the "brakes fail" when there is recessive loss of the TSC genes in specific cells. Mutations in tumor suppressor genes inactivate protein's function. In most cases, inactivation of both copies of the gene is required before a tumor is initiated. The germline mutation, or the "first hit," inactivates one copy of the gene in every cell in the body. A somatic mutation, or the "second hit," inactivates the remaining copy in a single cell, contributing to tumor formation. Often, the second hit somatic mutation is associated with the loss of the entire chromosomal region containing the gene, as well as many neighboring genes. This chromosomal loss is referred to as *loss of heterozygosity* (LOH).

The evidence supporting the role of *TSC1* and *TSC2* as tumor suppressor genes includes (1) that germline mutations in *TSC1* (van Slegtenhorst et al., 1997) and *TSC2* (European Chromosome 16 Tuberous Sclerosis Consortium, 1993) are predicted to inactivate protein function, and (2) that LOH in the *TSC1* or *TSC2* region occurs in TSC angiomyolipomas, rhabdomyomas, and astrocytomas (Carbonara et al., 1994; Green et al., 1994a; Bjornsson et al., 1996; Henske et al., 1996). However, it remains unclear whether somatic "second hit" mutations occur in cerebral cortical tubers. An early study did not find LOH in tubers (Henske et al., 1996), and a recent comprehensive search for LOH, as well as an exon-by-exon analysis for somatic mutations, did not reveal inactivation of the wild-type allele in cortical tubers (Niida et al., 2001). It seems likely, based on these studies, that the central nervous system (CNS) manifestations of TSC are the consequence of haploinsufficiency of tuberin or hamartin, in contrast to the renal and cardiac tumors, in which mutations inactivating both copies of the gene are indicated.

Approximately two-thirds of cases of TSC appear to result from de novo germline mutations. In these cases, a child with TSC carries a germline mutation that is not present in either of the parents, but will be transmitted to future generations. Numerous types of germline mutations are found in *TSC1* and *TSC2,* all of which are predicted to inactivate the function of hamartin or tuberin, respectively. Approximately 20 percent of germline *TSC2* mutations are missense changes (Jones et al., 1999; Dabora et al., 2001). Some of these occur at highly evolutionarily conserved residues and may indicate critical functional domains of tuberin. Specific mutations include the *TSC2* N1658K mutation, which was found to be highly ubiquitinated and unable to stabilize hamartin (Benvenuto et al., 2000); the P1675L GAP domain mutant; and the Y1571H mutation. These mutations appear to interfere with tuberin's phosphorylation and interaction

with hamartin (Aicher et al., 2001). In recent work, three mutations within the GAP domain (N1643K, N1651S, N1681K) (Soucek et al., 2001) and the P1675L (Khare et al., 2002) were found to have effects on the cell cycle and *p27* expression that were similar to those of wild-type *TSC2* (Soucek et al., 2001). The remaining 80 percent of *TSC2* mutations are either large deletions removing the entire gene, or smaller changes resulting in premature protein truncation. For reasons that are not yet understood, all identified *TSC1* mutations result in premature protein truncation, with no known missense mutations.

TSC has a wide range of phenotypic variability, with some individuals severely affected and others only mildly so. So far, no clear genotype-phenotype relationships have been established between mutations at specific residues or within particular domains and the clinical features of TSC. However, analysis of one large family with a germline missense mutation in *TSC2* suggests that such relationships may exist. The affected members of this family have a germline missense mutation in exon 34 of the *TSC2* gene (A4508C) that changes amino acid 1,503 from glutamine to proline. This amino acid is identical among the human, mouse, rat, *Fugu,* and *Drosophila* homologs of tuberin, suggesting that this amino acid is critical to the function of tuberin. Thirty-four members of this four-generation family (17 affected with TSC and 17 unaffected) underwent both physical and psychiatric assessments (Smalley et al., 1994). The majority of the affected individuals had mild physical expression of TSC, but there was significant clustering of neuropsychiatric disorders among affected individuals compared with their unaffected relatives. The disorders that were overrepresented in the affected individuals compared with their unaffected relatives included mood disorder, anxiety disorder, and autism. The largest difference was observed in anxiety disorder, which was seen in 10 of the affected individuals and in two of the unaffected individuals ($p = 0.016$). One affected child had pervasive developmental disorder and one had autism. Analysis of this family suggests that TSC can present phenotypically with mild physical signs and symptoms, but with significant neuropsychiatric disease, and that neuropsychiatric disease in TSC may be associated with specific missense mutations.

Recently, Dabora et al. (2001) conducted a mutational and clinical study of 224 TSC patients that revealed clear differences between the severity of *TSC1* and *TSC2* disease. Among these TSC patients, mutations were identified in 186 (83%) of the cases, comprising 158 in *TSC2* and 28 in *TSC1.* This confirmed earlier, smaller studies in which the frequency of germline *TSC2* mutations was several fold higher than germline *TSC1* mutations. Using a standardized clinical assessment covering 16 TSC signs, de novo *TSC1* mutations resulted in milder disease, on average, compared with de novo germline *TSC2* mutations, although there was considerable overlap between the groups. In aggregate, *TSC1* patients had a

lower frequency of seizures, less severe mental retardation, fewer subependymal nodules and cortical tubers, less severe kidney involvement, no retinal hamartomas, and less severe facial angiofibroma. Some clinical features, including the more severe kidney cysts or angiomyolipomas, forehead plaques, retinal hamartomas, and liver angiomyolipomas, were very rare or not seen at all in *TSC1* patients (Dabora et al., 2001). Patients in whom no mutation was found also had disease that was milder, on average, than that in patients with *TSC2* mutations, and was somewhat distinct from patients with *TSC1* mutations.

CELLULAR FUNCTIONS OF HAMARTIN AND TUBERIN

Multiple lines of evidence from both mammalian cells and *Drosophila* indicate that tuberin and hamartin regulate cell growth, but the exact in vivo mechanisms of this growth regulation and their contribution to the clinical manifestations of TSC are not completely understood. Both gene products, hamartin and tuberin, are found in the normal adult brain, kidney, and heart, with their expression evident in cortical neurons, renal tubular epithelial cells, pancreatic islet cells, bronchial epithelial cells, and pulmonary macrophages (Plank et al., 1999). Tuberin and hamartin physically interact (Plank et al., 1998; van Slegtenhorst et al., 1998), suggesting that they function in the same cellular pathway(s). Hamartin also stabilizes tuberin by protecting it from ubiquitin-mediated degradation (Benvenuto et al., 2000). The tuberin-hamartin interaction is consistent with the similar clinical manifestations of *TSC1*- and *TSC2*-linked disease (Dabora et al., 2001).

Mammalian tuberin and/or hamartin appear to be involved in at least four cellular pathways: vesicular trafficking, cell cycle regulation, cell adhesion via the small GTPase Rho, and steroid hormone function. The in vivo relevance of these pathways to the clinical manifestations of TSC is an area of active investigation. The evidence supporting each of these pathways is briefly summarized here.

Tuberin has GAP activity for Rab5, and cells lacking tuberin have abnormal endocytosis (Xiao et al., 1997). Tuberin also has GAP activity for Rap1 (Wienecke et al., 1995). Tuberin appears to be required for the correct membrane localization of polycystin, the product of autosomal dominant polycystic kidney disease (ADPKD) gene, *PKD* (Kleymenova et al., 2001). It is not known whether this localization requirement is related to aberrant vesicular trafficking.

Tuberin (Soucek et al., 1997; Ito and Rubin, 1999) and hamartin (Miloloza et al., 2000; Tapon et al., 2001) have been shown to regulate the G1 phase of the cell cycle. However, recently, it was shown that overexpression of mutant forms of tuberin (containing patient-derived mutations) can also regulate G1 (Soucek et al., 2001; Khare et al., 2002).

Hamartin interacts with the ezrin-radixin-moesin (ERM) family of cytoskeletal proteins, activates the GTPase Rho, and regulates focal adhesion formation (Lamb et al., 2000).

The carboxy-terminus of tuberin interacts with steroid hormone receptors as a coactivator (Henry et al., 1998). Tuberin binds calmodulin, and calmodulin binding is required for tuberin's modulation of steroid receptor function (Noonan et al., 2002).

Autism and TSC

Autism is a common behavioral feature of TSC, as are other neurobehavioral and psychiatric disorders, including attention deficit hyperactivity disorder (ADHD) and anxiety (for a review, see Smalley et al., 1992). Estimated rates of autism in TSC range from 17 to 68 percent (Smalley et al., 1992; Gonzalez et al., 1993; Hunt and Shepherd, 1993; Gillberg et al., 1994; Bolton and Griffiths, 1997; Baker et al., 1998; Gutierrez et al., 1998). The variability in estimated rates of autism in TSC appears to be a function of the methods used to define autism, the variability in TSC samples (particularly in terms of comorbid mental retardation), and methods of ascertainment of TSC cases (for a review, see Smalley, 1998). Two studies used the highly reliable Autism Diagnostic Interview (ADI) (Le Couteur et al., 1989) for assessing autism in series of 28 TSC patients (Gutierrez et al., 1998) and 20 TSC patients (Baker et al., 1998). Rates of autism in TSC using this structured interview were 27 percent and 20 percent in the two studies, respectively. Similar rates were obtained in two other studies when DSM-III-R and ICD-10 criteria were applied to TSC patient populations. Gonzalez and colleagues (1993) found a rate of 26 percent using DSM-III-R criteria in 27 TSC clinic cases, whereas Bolton and Griffiths (1997) found that 22 percent of 19 TSC clinic cases met ICD-10 criteria for autism. Overall, the systematic evaluation of TSC cases using structured interviews and/or rigorously applied diagnostic criteria suggests that the rate of autism within TSC is approximately 20–27 percent. An association of autism and TSC is evident by the elevated rates of autism among TSC cases. Similarly, the frequency of TSC is elevated among individuals with autism, particularly among those with seizure disorders. An accurate estimate of the frequency of TSC in autism is difficult to determine, because the medical diagnosis of TSC often precludes further investigation of the case in autism studies. In studies in which the rates of TSC within autism samples are reported, the estimated rate ranges from 1 to 4 percent (reviewed in Smalley et al., 1992; see also Gillberg and Coleman, 1992). Furthermore, the frequency of TSC among the subgroup of autistic individuals with a comorbid seizure disorder appears to be significantly greater than the 1–4 percent observed for the group as a whole. In

two studies of 24 (Riikonen and Amnell, 1981) and 66 (Gillberg, 1991) autistic or autistic-like cases with a seizure disorder, the number of TSC cases was two (8%) and nine (14%), respectively, suggesting that among autistic individuals with seizures, TSC is a common occurrence. Overall, the data support a strong association of TSC and autism.

Mechanisms of Comorbidity

We previously described three alternative hypotheses of the observed association of autism and TSC (Smalley, 1998). Briefly, these are (1) secondary effects of the TSC gene, such as the development of infantile spasms, mental retardation, and/or cortical tubers; (2) a susceptibility gene underlying autism either in linkage disequilibrium with a TSC gene and/or interaction of its gene product with the TSC gene product in the pathogenesis of autism or related disorders; (3) disruption of TSC gene function in early stages of neurogenesis, leading to abnormal brain development in those regions involved in autism.

Hypothesis 1 is supported by several findings. First, there are elevated rates of autism among individuals with infantile spasms (IS) in the absence of TSC (Riikonen and Amnell, 1981). Second, successful treatment of IS in TSC cases can significantly reduce behavioral sequelae of autism (Jambaque et al., 2000). Third, the location of cortical tubers, particularly in the temporal lobe, is associated with autism (Gonzalez et al., 1993; Bolton and Griffiths, 1997; Smalley, 1998). However, in contrast to the expectations of this hypothesis, TSC cases with IS have rates of autism significantly higher than is found for IS samples alone (Riikonen and Amnell, 1981), and the specificity of cortical tuber location and autism is less than perfect (for a review, see Smalley, 1998). Furthermore, autism and other neuropsychiatric disorders occur in TSC cases in the absence of seizures and mental retardation (Smalley et al., 1994; Smalley, 1998). These data suggest that IS, mental retardation, and cortical tuber locations may contribute to the elevated rates of autism in TSC, but that another underlying mechanism is likely to account for the observed association.

Hypothesis 2 is supported by several recent genome-wide scans of autism. In three scans completed to date, a region on 16p, near the *TSC2* locus, is indicated by modest linkage peaks. In the first genome-wide scan for autism conducted by the International Molecular Genetic Study of Autism Consortium (1998) and in a follow-up study by that group in 2001, the region on 16p13 near marker D16D3012 yielded a multipoint maximum logarithm of the odds (LOD) score (MLS) of 2.9. In a study by Liu et al. (2001) with multiplex families for autism, an MLS of 2.19 was observed near marker D162619, and in an earlier study by Philippe et al. (1999), a nominal MLS of .74 was seen with marker D16S3075 near

the other two markers. In a recent investigation of 277 affected sibling pairs with ADHD, we found significant evidence for linkage in this same region on 16p13 near marker D16S3114 with a multipoint MLS of 4.2 (Smalley et al., 2002). The 1-LOD support interval highlighted by the ADHD scan and including the maximum linkage peaks in the autism studies spans approximately 7 Mb and is centromeric to the *TSC2* locus by approximately 5 Mb, based on physical databases. Linkage disequilibrium would be unlikely if the estimated location for a putative risk gene in ADHD and/or autism are accurate; however, given the difficulty in fine mapping for a highly heterogeneous trait like autism or ADHD, until the risk locus (or loci) is identified, further investigations of the *TSC2* locus and adjacent regions are warranted. A second possibility is that the *TSC2* product, tuberin, interacts with the putative risk gene product underlying autism and/or ADHD. A mechanism of this sort is evident for tuberin and polycystin, the gene product of the polycystic kidney disease gene (*PKD1*), located adjacent to *TSC2*. Tuberin is required for the correct membrane localization of polycystin (Kleymenova et. al., 2001), and thereby tuberin modifies polycystin's function. In a similar way, the gene product of a putative risk locus centromeric to *TSC2* may interact with tuberin in gene expression through its role in vesicular trafficking, cell cycle regulation, or some other mechanism. These data suggest that a putative risk gene in autism and ADHD is in close proximity to the *TSC2* locus and that the association of these disorders and *TSC2* may occur due to linkage disequilibrium or a gene × gene interaction.

Hypothesis 3 is supported by two lines of research. First, a specific mutation in the *TSC2* locus is associated with significant psychopathology, including autism, in a large extended family, as previously described (Smalley et al., 1994; Khare et al., 2001). Second, the CNS abnormalities observed in TSC appear to be due to haploinsufficiency rather than to the double-hit gene loss observed in non-CNS tissue (see above). In contrast, linkage findings in autism are not supportive of this hypothesis, because the linkage peaks in autism at 16p are centromeric of *TSC2*. However, further investigations of the behavioral sequelae of TSC and underlying CNS dysfunction are warranted. If the haploinsufficiency of tuberin or hamartin underlies abnormal CNS development in TSC, it is feasible that abnormal cell orientation and/or dysregulation of cell function may occur in the CNS without the manifestation of cortical tubers and/or subependymal nodules (i.e., morphometric and/or functional CNS abnormalities may be present but not detectable by the traditional magnetic resonance imaging/computed tomography scans used in TSC diagnosis). Currently, brain-behavioral investigations in TSC have largely focused on the location and size of cortical tubers and not on morphometric and/or functional changes.

Taken together, the data currently available cannot exclude hypothesis 1 or 3, but perhaps best support hypothesis 2. The recent converging evidence from genome-wide scans in autism and ADHD suggests that this region is highly likely to harbor a risk gene or genes contributing to these two behavioral disorders, the most common psychiatric disorders observed in TSC. A plausible model underlying the observed association of TSC and autism, as well as ADHD, may be an interaction of tuberin with the gene product(s) of the putative risk locus (or loci). Future work investigating the regulation of tuberin and/or its regulation of other genes potentially involved in neurogenesis and implicated by positional cloning in multiplex families with autism and/or ADHD is warranted.

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21

The Roles of Dopamine and Norepinephrine in Autism: From Behavior and Pharmacotherapy to Genetics

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Autism spectrum disorders (ASD) are complex neurodevelopmental disorders with a wide range of behavioral manifestations. Recent studies indicate that the deleterious developmental and behavioral sequelae of autism can be minimized in some children through intensive behavioral intervention (Lovaas, 1987; McEachin et al., 1993; Smith et al., 1997; Filipek et al., 2000), especially during early childhood. Thus, early diagnosis is essential. Clearly, knowledge about the etiology of these conditions will lead to a better understanding of how to develop optimal treatments for this heterogeneous group of disorders.

As discussed elsewhere in this book, behavioral, neuropathological, neurochemical, and pharmacotherapeutic findings in autism often vary from one study to the next, likely reflecting the etiologic heterogeneity of ASD. There are no consistent findings for all individuals with autism. At best, a certain percentage of individuals shows a particular finding in several studies: for example, approximately 30 percent of individuals with autistic disorder have hyperserotonemia. For other studies, the number of individuals assessed is small. Given the heterogeneous phenotype in persons with ASD, and even within the most extreme group of subjects with autistic disorder, different results among studies are not unexpected. Bias of ascertainment and biases in physician referrals (e.g., individuals with autism referred to a researcher interested in obsessive compulsive disorders may differ considerably from those made to a researcher studying autism and developmental disability) may contribute to differences in outcomes of a host of studies, including imaging, neuropathology, neurochemistry, and drug responses. It is clear that ASDs are heterogeneous not only in presentation, but also in etiology. And, despite the clear evidence for a strong genetic component to the etiology of ASDs, the lack of uniform findings among studies and the

association of autism with specific genetic syndromes (e.g., tuberous sclerosis, fragile X syndrome, phenylketonuria [PKU]), indicate that ASDs are also genetically heterogeneous. In contrast to the concerns about the variable and "inconsistent" findings in brain morphology, neurochemistry, and responses to pharmacotherapy, we believe that these findings should be embraced and followed up, as they may provide the necessary clues to the several genes that are likely involved in producing the variable phenotypes seen in persons with ASD.

Catecholamines have been examined extensively in ASD, because of their involvement in behavior disturbances seen in ASD (e.g., motor control, reward, cognition, stereopathies, obsessional relations with objects, hyperactivity, attention). This chapter briefly reviews some of the evidence in support of a dopaminergic and/or noradrenergic role in the etiology or modification of the phenotype in ASD. It begins with a brief summary of some of the neurobiologic, neurochemical, and pharmacotherapeutic findings in autism, followed by a review of genetic studies on genes involved in the synthesis, metabolism, and function of dopamine and norepinephrine.

Biologic Findings: Role of Dopamine and Norepinephrine

NEUROBIOLOGY

Abnormal brain structure and function are common in autism, but there is variability among subjects. Postmortem findings have shown reduced cell size and increased cell packing density in the amygdala, hippocampus, entorhinal cortex, mammillary body, medial septal nucleus, and anterior cingulate in some individuals (Bauman, 1996; Kemper and Bauman, 1998); although another study failed to find increased hippocampal neuronal density (Bailey et al., 1998). Reduced numbers of Purkinje cells and the continued presence of olivary neurons suggest an early prenatal origin for autism (Bauman, 1996).

There are several reports of postnatal macrocephaly in autism (Bolton et al., 1994; Bailey et al., 1995; Woodhouse et al., 1996; Fidler et al., 2000; Miles and Hillman, 2000; Courchesne et al., 2001; Aylward et al., 2002). Qualitative magnetic resonance imaging (MRI) scans suggest neuronal migration defects in some cases of autism (Piven et al., 1990). Enlarged occipital and parietal lobes have also been reported (Piven et al., 1995, 1996; Filipek, 1996). Among explanations for macrocephaly in autism are increased neurogenesis, decreased neuronal cell death, and increased production of nonneuronal tissue. Because neurotransmitters are involved in both the differentiation and the maintenance of neurons, their over- or underproduction or availability could influence the numbers of specific neurons in different regions of the brain.

NEUROCHEMISTRY

In the past, neurochemical studies generally focused on the dopaminergic, serotinergic, and peptidergic systems in autism. Examinations of homovanillic acid (a major metabolite of dopamine) in cerebral spinal fluid (Cohen et al., 1974, 1977; Gillberg et al., 1983; Klykylo et al., 1985; Gillberg and Svennerholm, 1987; Narayan et al., 1993) and urine (Launay et al., 1987; Garreau et al., 1988; Martineau et al., 1994; Hameury et al., 1995) have produced conflicting results. A study of norepinephrine metabolites failed to show abnormalities (Anderson and Hoshino, 1997). Again, the variation in findings could reflect the relatively small numbers of individuals studied in some cases, particularly given the heterogeneous nature of autism.

PHARMACOTHERAPY

Many pharmacologic agents have been used, targeting various behaviors in persons with autism. Amelioration of symptoms with some, and aggravation with others, can provide clues to defects in autism. Haloperidol (Anderson et al., 1984, 1989; Campbell et al., 1988; Perry and Grimes, 1989) and pimozide (Naruse et al., 1982; Ernst et al., 1992), both dopamine blockers, have been found to reduce withdrawal symptoms, hyperactivity, negativism, and stereotypies, whereas stimulants, such as dextroamphetamine (which enhances the effects of dopamine) generally exacerbates symptoms (Campbell et al., 1972). The newer neuroleptics, such as risperidone, which block both dopamine (D_2) and serotonin ($5HT_2$) receptors, lead to clinical improvement (Fisman and Steele, 1996; Hardan et al., 1996; Findling et al., 1997; McDougle et al., 1997; Perry et al., 1997). Such findings argue for a role for dopamine in autism. Clonidine is an α_2-adrenergic receptor agonist that appears to reduce hyperactivity, impulsivity, and irritability (Fankhauser et al., 1992) and propranolol (a β-blocker) has been shown to be successful in the treatment of aggression and impulsivity in autistic adults (Ratey et al., 1987). These findings argue for a role of the noradrenergic system in autism or some of the behaviors that are often present in persons with autism.

Gene Studies

The following sections describe the results of a series of genetic studies, carried out on individual genes within pathways for the synthesis, metabolism, and use of dopamine and norepinephrine. Our own studies have been stimulated by the observation of Dr. Paula Robinson, in her M.S. studies at McMaster University, Ontario, that mothers of autistic children had a higher frequency of alleles asso-

ciated with lower dopamine β-hydroxylase activity (Robinson et al., 2001). This led to the novel hypothesis that maternal genes may influence early development of the fetal brain, and that suboptimal exposure of the fetal brain to maternal dopamine and norepinephrine levels may lead to susceptibility to autism (Robinson et al., 2001). We, and others, have proposed that complex genetic disorders, such as ASD, may result from an interaction of alleles at several loci. On their own, these alleles (normal variations in the population) may have subtle or no phenotypic effects, but, in combination, they may lead to the extreme phenotype of autism. If alleles at, for example, five or six genes are necessary to produce autistic disorder, then the presence of only three or four of these risk alleles may lead to a milder form of pervasive developmental disorder (PDD). Furthermore, if alleles at different sets of five or six genes from a total of 15 or so genes results in the general phenotype of autistic disorder, then different combinations might produce different phenotypes. Such a "threshold" model could explain why affected brothers are not always concordant for the subtype of ASD (Holden et al., 1996) and how other forms of ASD are found in families with children with autistic disorder (Le Couteur et al., 1989; Bailey et al., 1995, 1998). The overall risk of ASD to siblings of PDD probands is 5–6 percent, with 20 percent of siblings of autistic probands having communication or social impairments or restricted interests (versus 3% of siblings with Down syndrome) (Bolton et al., 1994), indicating that susceptibility to autism may also confer susceptibility to milder manifestations of PDD, including some falling below the threshold for a diagnosis of ASD.

Based on this reasoning, we have been studying genes involved in the synthesis and metabolism of dopamine and norepinephrine, expecting that some families will have an accumulation of higher- or lower-expressing alleles in some of the enzymes in this pathway or in the transporter proteins or receptors that control availability of these neurotransmitters, and that the additive or multiplicative effects of these functional alleles lead either to susceptibility to autism or to modification of the phenotype in individuals with ASD. Here we present the findings of various researchers, together with our own findings to date, on these relevant genes. For our own findings, note that initial studies on each of the genes have been carried out on 37 multiplex (MPX) families (called Set I), all referred to one physician. The second set of 81 MPX families (Set II) consists of families recruited through the Autism Genetic Resource Exchange (AGRE) (Geschwind et al., 2001; Liu et al., 2001); these are volunteer families. Of these, 40 families had two affected sons; 32 families had one affected son and one affected daughter; seven families had two affected daughters; one family had three affected sons; and one family had three affected daughters. Among the 81 families, 48 had two children with autism, 26 had one child with autism and one

child with PDD, three had one child with autism and one with Asperger syndrome, two had two children with autism and one child with PDD, one had three children with autism, and one had two children with PDD. All affected children were assessed using the Autism Diagnostic Interview–Revised (ADI-R) (Lord et al., 1994) and the Autistic Diagnostic Observation Schedule (ADOS) (Lord et al., 1989) or ADOS–Generic (Lord et al., 2000). Both sets of families were unselected for stereotypies and other psychiatric behaviors and disorders that may be associated with autism, such as hyperactivity. The comparison group samples, consisting of 197 male and 190 female subjects, were randomly picked from heel prick blood spots from anonymous neonates, obtained through the Ontario Ministry of Health—taken for the purpose of newborn PKU testing.

GENES INVOLVED IN THE SYNTHESIS OF DOPAMINE AND NOREPINEPHRINE

Dopamine is synthesized from tyrosine, via tyrosine hydroxylase and L-aminoacid dopa-decarboxylase. Norepinephrine is synthesized from dopamine by dopamine-β-hydroxlase (DBH). Because tyrosine is synthesized from phenylalanine by phenylalanine hydroxylase (PAH), and defects in this gene result in phenylketonuria, which is often associated with autism, we begin this report with results on the *PAH* gene.

Phenylalanine Hydroxylase. The *PAH* gene is located on chromosome 12, at band q24. Mutations in this gene result in classical PKU. Affected individuals have blond hair, blue eyes, microcephaly, hyperactivity, self-injurious behavior, irritability, marked social impairments, and epilepsy. Persons with PKU, who maintain a phenylalanine-free or restricted diet from shortly after birth until the age of 10–12 years, have average IQs, although these may be lower than expected based on sibling and parent IQs (Holtzman et al., 1986). It has long been recognized that persons with PKU who are not treated with dietary restrictions show autistic features, which are diminished considerably when reduced-phenylalanine diets are implemented (Miladi et al., 1992). A study of cognitive patterns in children with high-functioning autism and those with poorly controlled PKU showed that both groups had similar profiles of lower comprehension when compared on a test of block design, and the investigators suggested that dopamine deficiency may be responsible for these findings (Dennis et al., 1999).

Despite the very considerable amount of information that is available on the *PAH* gene with respect to the locations and nature of mutations causing PKU, there have been no descriptions of functional polymorphisms in this gene. We therefore chose to study a tetranucleotide microsatellite repeat locus in intron 3

of the *PAH* gene in 128 multiplex families with ASD. In the initial set of 37 families, we found a significantly increased frequency of the 248-bp allele and a decreased frequency of a 240-bp allele in mothers of affected children compared to female controls (Figure 21.1A). When the studies were extended to Set II families, there was a significant increase in the frequency of the PAH-248 allele, but the frequency of the PAH-240 allele was similar to controls (Figure 21.1B). Indeed, based on the results in the initial set of families, in which the 240-bp allele appears to be protective and the 248-bp allele appears to be a risk allele, one would expect few mothers to have the 240/248 genotype, which is the case (two of 13 mothers with a 248-bp allele have a 240-bp allele, or 15%). However, in the second set of families, there is a higher frequency of mothers with this genotype among those with a 248-bp allele (10 of 28, or 36%), indicating differences in the composition of the two sets of families. The increased frequency of the 248-bp allele in both sets of families suggests that *PAH* acts as a maternal effect locus, affecting the development of those children who are genetically at risk for ASD. To support this notion, we found that the children whose mothers had at least one PAH-248 allele had more severe verbal communication deficits ($\chi^2 = 5.81$; $df = 1$; $p < 0.02$).

Thus, our findings suggest that the *PAH* locus may affect development of children at risk through a maternal effect in a subset of families. This finding is intriguing, given the recognized teratogenic effects of maternal PKU, in which children born to mothers with PKU who are not on a reduced phenylalanine diet during pregnancy are at very high risk for congenital abnormalities. At this time, we do not know whether the PAH-248 allele is associated with altered *PAH* activity, but these studies are in progress.

Tyrosine Hydroxylase. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of dopamine and norepinephrine. The TH gene is located on the short arm of chromosome 11 at 11p15 and has been tested as a candidate gene in a number of different populations, including schizophrenia, in which a rare allele (Ep) in a tetranucleotide repeat within intron 1 was found to be more frequent in affected persons than in controls (Meloni et al., 1995; Thibaut et al., 1997). Philippe et al. (2002) examined this repeat in 38 MPX families and a control population and found no evidence for this gene as a susceptibility gene for ASD. However, more recent studies by our group found an increased frequency ($\chi^2 = 5.852$; $df = 1$; $p = 0.016$) of the 5-repeat allele in individuals with ASD in a study of 37 families with two or more cases (Figure 21.2). Furthermore, there was a trend toward increased transmission of this allele to affected children as determined using a family-based association test (FBAT; $p = 0.07$) in these families (Table 21.1). Although these findings appeared promising, in a replication study,

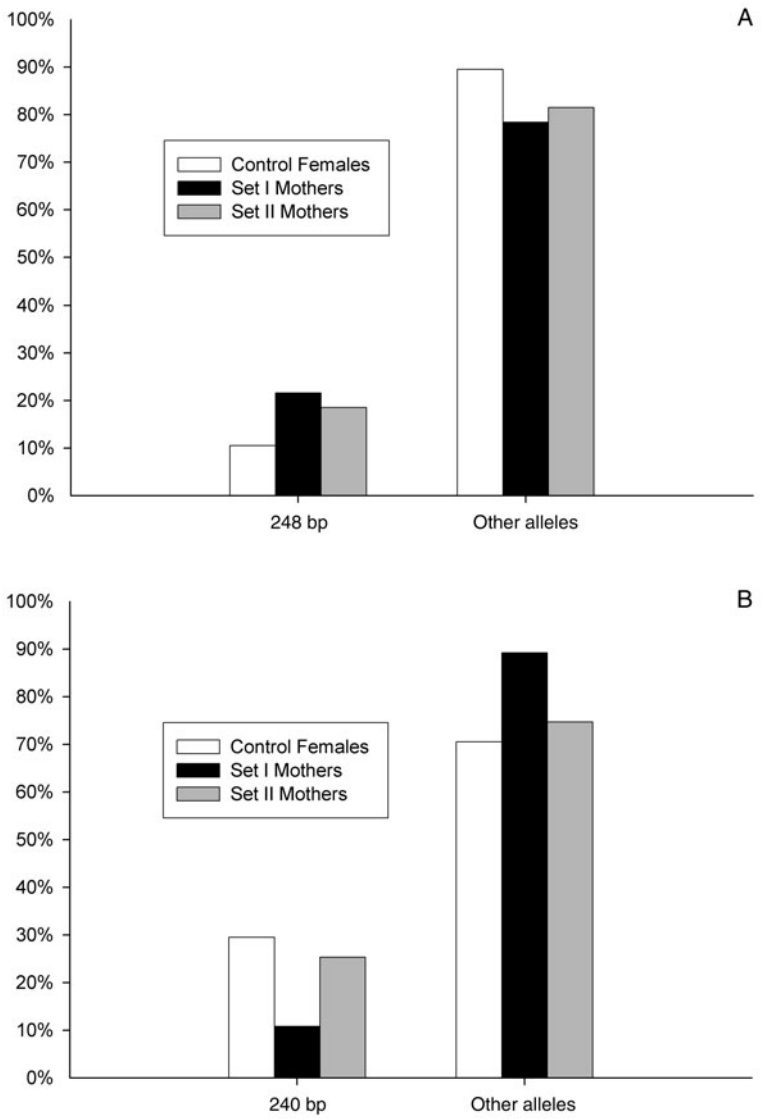

FIGURE 21.1. Comparisons of frequencies of *PAH* alleles in mothers from two cohorts of families with autism and a comparison group. (A) Distribution of PAH-248 and other alleles in mothers of autistic children and a female comparison group (Set I: $\chi^2 = 3.847$; $df = 1$; $p = 0.050$; Set II: $\chi^2 = 3.951$; $df = 1$; $p = 0.047$). (B) Distribution of PAH-240 and other alleles in mothers of autistic children and a female comparison group ($\chi^2 = 8.034$; $df = 1$; $p = 0.005$; $\chi^2 = 0.566$; $df = 1$; $p = 0.452$).

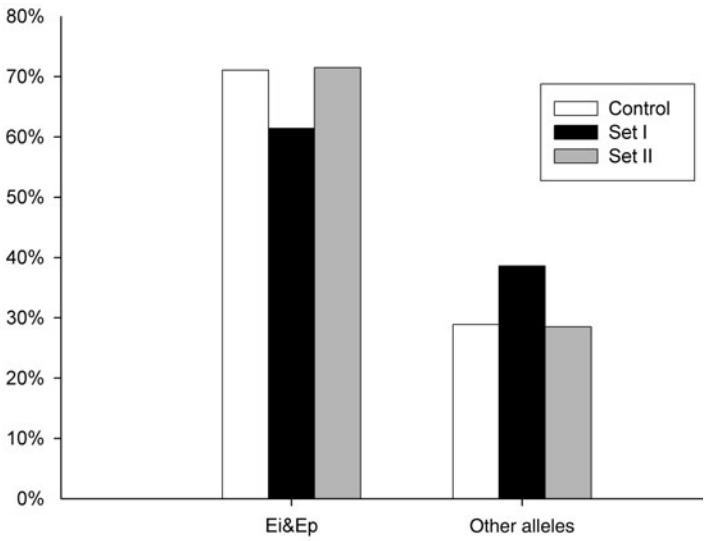

FIGURE 21.2. Comparisons of frequencies of *TH-STR* palleles in two cohorts of individuals with autism and a comparison group. Distribution of THPAH- Ei and Ep alleles versus other alleles in affected individuals and controls (Set I: $\chi^2 = 5.851$; $df = 1$; $p = 0.016$; Set II: $p = 1.000$).

we did not find any differences in the distribution of alleles in the family members and controls.

L-Amino Acid Dopadecarboxylase. The L-amino acid dopadecarboxylase (*LAADC*) gene is located on chromosome 7 at position p12.1–p12.3 (Sumi-Ichinose et al., 1992). This enzyme is involved in the biosynthesis of both dopamine and serotonin and is rate-limiting in the synthesis of 2-phenylethylamine, which positively modulates dopaminergic transmission. It has therefore been screened for polymorphisms and tested as a candidate gene for both schizophrenia and bipolar disorder. No evidence was found to support the *LAADC* gene as a candidate gene for either disorder (Speight et al., 2000). We examined the four base-pair insertion/deletion polymorphism in the 5′UTR of the neuronal specific messenger RNA (mRNA) (Speight et al., 2000) and found a trend toward an increased frequency of the deletion variant in the mothers of autistic children in the initial families tested ($N = 37$ MPX; $\chi^2 = 3.59$; $df = 1$; $p < 0.06$), with a relatively high proportion of homozygous *LAADC*-del/del genotypes in these mothers compared to controls (19.4% versus 6.6%; $\chi^2 = 6.4$; $df = 2$; $p = 0.04$) (Figure 21.3). Again, these findings were not replicated when the studies were expanded to a larger number

TABLE 21.1. Family-Based Association Test (FBAT) Analysis of *TH* in Set I Families

Allele	*afreq*	*Family Number*	*S*	*E(s)*	*Var(S)*	*Z*	*p*
A	0.261	24	32	34.9	14.147	–0.773	0.4397
B	0.16	14	18	17.4	8.397	0.205	0.8377
C	0.088	12	12	13.5	6.75	–0.577	0.5637
D	0.162	18	23	26	10	–0.949	0.3428
Ei	0.312	23	40	33.2	14.402	1.795	0.0726
Ep	0.017	1					

of families, possibly reflecting differences in the two groups of families studied. However, there was an increased frequency of affected children with the 208/208 genotype when only families with affected sons were considered ($\chi^2 = 4.404$; $df = 1$; $p = 0.04$), but there was no correlation of this genotype with differences in any of the three domains of autism as determined in the ADI-R.

Dopamine β-hydroxylase. The DBH gene is located at band q34 on chromosome 9, very close to one of the genes for tuberous sclerosis, a disorder frequently associated with autistic behaviors (Hunt and Dennis, 1987). Robinson et al. (2001) initially examined a 19-base-pair insertion/deletion polymorphism in this gene in Set I families. We found an increased frequency of the deletion alleles ($\chi^2 = 4.39$; $df = 1$, $p < 0.04$) (Figure 21.4) in mothers with autistic children and suggested that this gene may act as a maternal effect locus, resulting in a suboptimal intrauterine environment that adversely affects early brain development in genetically susceptible fetuses. Our studies suggested that the deletion/insertion polymorphism, or a locus nearby and in linkage disequilibrium with it, was associated with DBH enzyme activity, such that deletion homozygotes had the lowest DBH activity and insertion homozygotes had the highest DBH enzyme activity, with heterozygotes having intermediate activity. A study by Zabetian et al. (2000) provided evidence that a C/T polymorphism 3 kb 5′ to the *DBH* transcriptional start is a functional polymorphism in this gene, with the C-variation being correlated with higher activity of the enzyme. We therefore extended our study to include both polymorphisms in the original families, as well as in Set II families. Although we did not find support for the 19-bp deletion alleles being more frequent in mothers, we did find that the T-allele was more frequent in fathers in Set II families ($\chi^2 = 5.378$; $df = 1$; $p = 0.02$). We further found that the

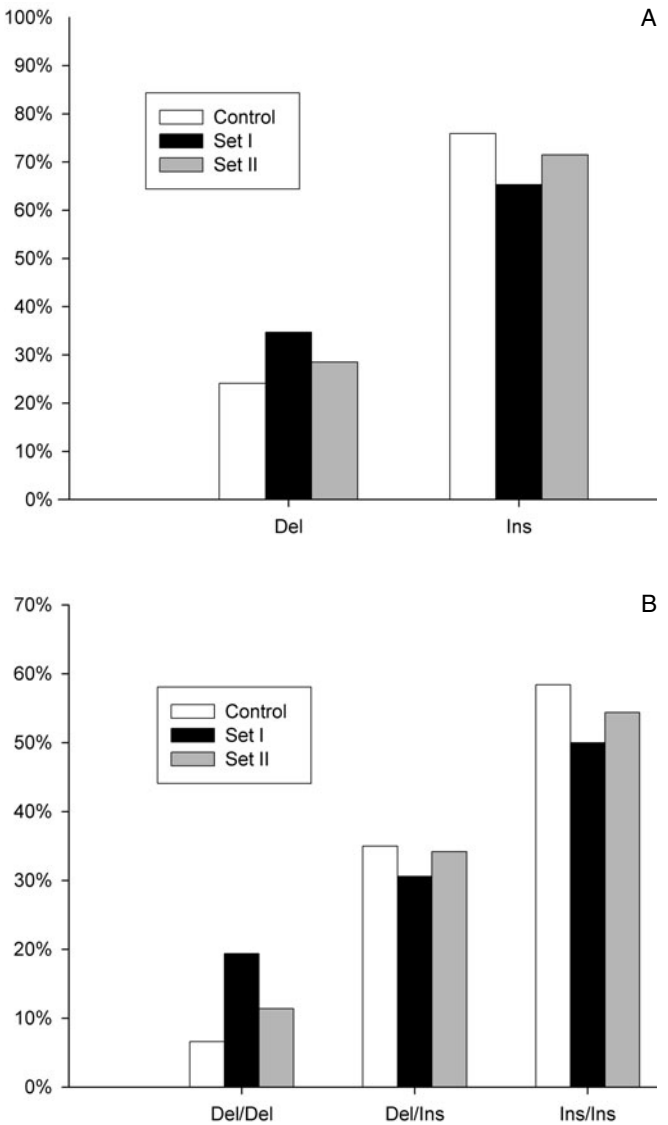

FIGURE 21.3. Comparisons of frequencies of *LAADC* alleles in mothers from two cohorts of families with autism and a comparison group. (A) Distribution of *LAADC* ins/del alleles in mothers of autistic children and a comparison group (Set I: $\chi^2 = 3.585$; $df = 1$; $p = 0.058$; Set II: $\chi^2 = 1.137$; $df = 1$; $p = 0.286$). (B) Distribution of *LAADC* ins/del genotypes in mothers of autistic children and a comparison group ($\chi^2 = 6.042$; $df = 2$; $p = 0.041$; $\chi^2 = 1.790$; $df = 2$; $p = 0.409$).

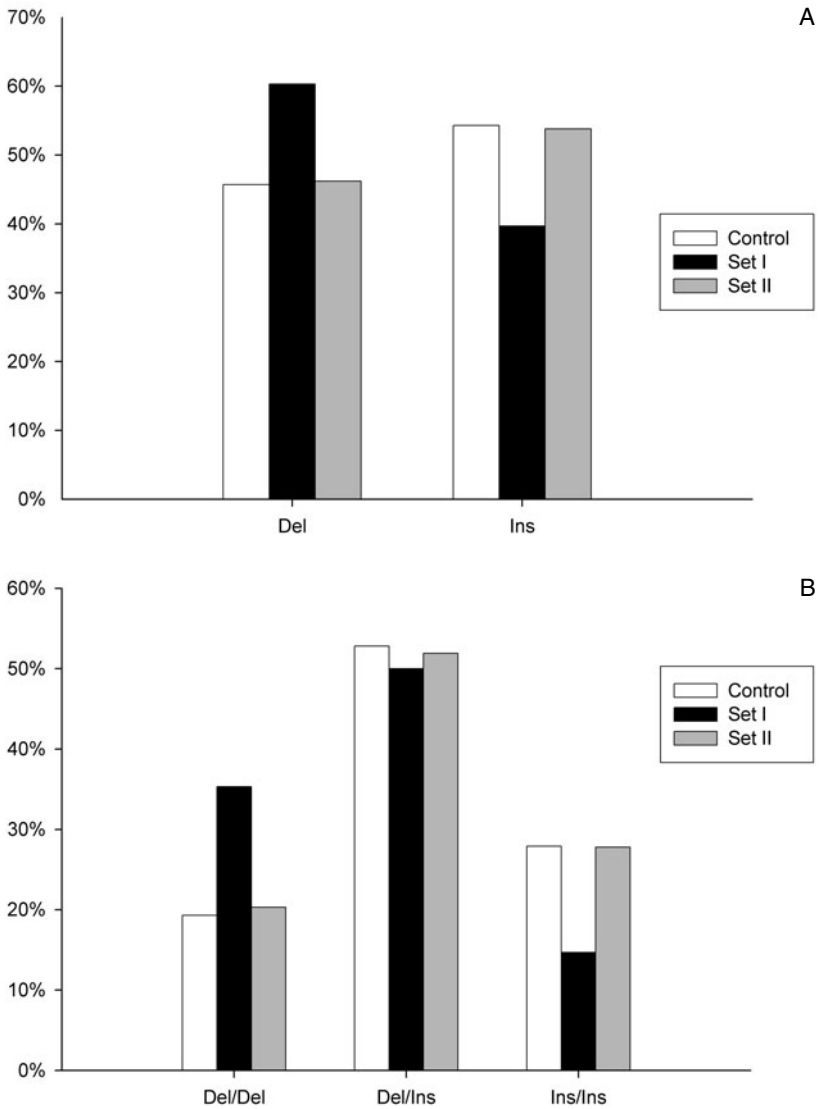

FIGURE 21.4. Comparisons of frequencies of *DBH* alleles in two cohorts of families with autism and a comparison group. (A) Distribution of *DBH* ins/del alleles in mothers of autistic children and a comparison group (Set I: $\chi^2 = 4.392$; $df = 1$; $p = 0.036$; Set II: $\chi^2 = 0.000$; $df = 1$; $p = 1.000$). (B) Distribution of *DBH*-ins/del genotypes in mothers of autistic children and a comparison group (Set I: $\chi^2 = 5.423$; $df = 2$; $p = 0.066$; Set II: $\chi^2 = 0.035$; $df = 2$; $p = 0.982$). (C) Distribution of *DBH* C/T alleles in fathers of autistic children and a comparison group (Set I: $\chi^2 = 1.479$; $df = 1$; $p = 0.224$; Set II: $\chi^2 = 5.378$; $df = 1$; $p = 0.020$). (D) Distribution of *DBH* SNP genotypes in fathers of autistic children and a comparison group (Set I: $\chi^2 = 2.122$; $df = 2$; $p = 0.346$; Set II: $\chi^2 = 6.885$; $df = 2$; $p = 0.032$).

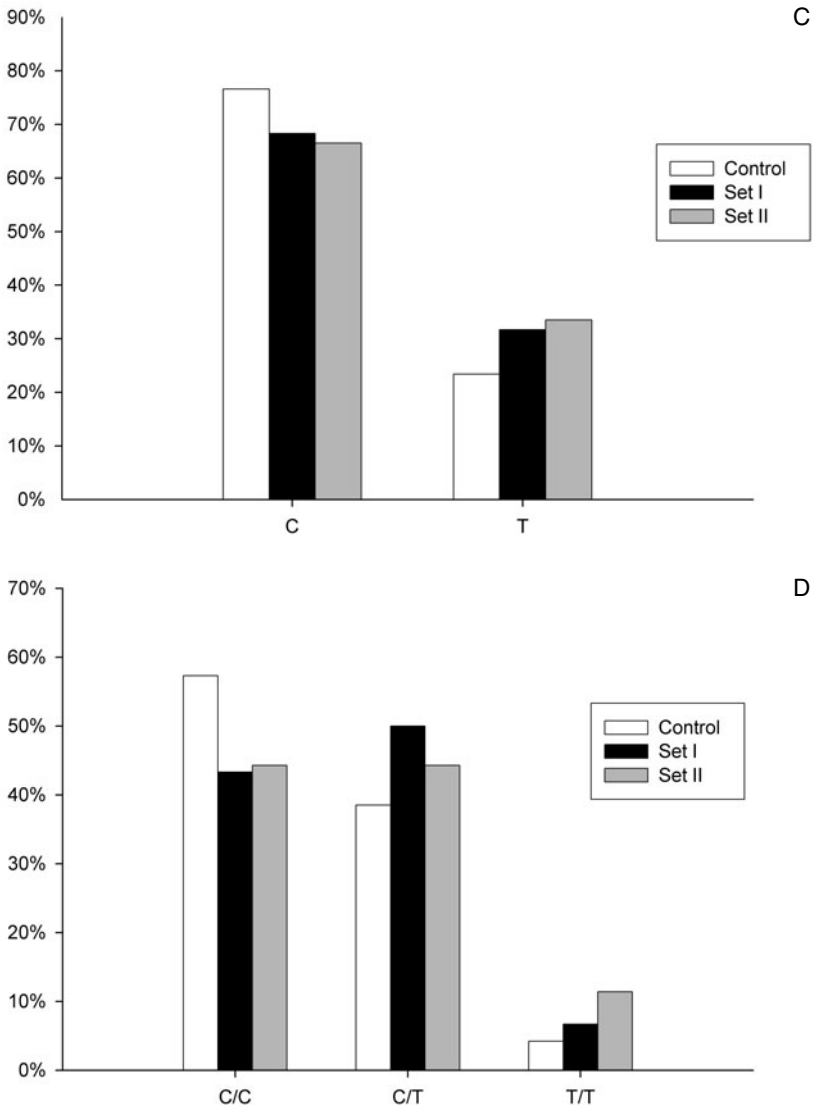

FIGURE 21.4. *Continued*

children of fathers homozygous for the T-allele were significantly more impaired in reciprocal social interaction.

It is difficult to reconcile these disparate findings, although differences in the two groups of families may well account for them. The first group of families had a high proportion of affected males (30 families with two affected males, a single family with two affected females), and there were few unaffected male siblings in

these families. The second set of families was recruited through the AGRE (Geschwind et al., 2001; Liu et al., 2001), and included volunteer families, with proportionately fewer families with two affected males. In addition, a large proportion of the families had unaffected male siblings. This point is important if we consider that the original family set had few unaffected males and showed an apparent maternal effect for the *DBH* insertion/deletion polymorphism—based on a maternal effect hypothesis, we would expect few unaffected males in such sibships. Given the various findings we obtained on the Set I families, we think that these may be a more homogeneous group than other groups of families. Curiously, Mankowski et al. (2002) examined both of these polymorphisms in families with autistic children and found no difference in the frequencies of the 5′ C and T alleles or genotypes in mothers, but did find an increased frequency of mothers with the *DBH*+/*DBH*+ genotype. They also found that the children of these mothers scored poorer on measures of obsessive compulsive behaviors than did children of mothers with the *DBH*–/*DBH*– genotype (0.33 versus 0.21; $p = 0.001$). These authors concluded that the *DBH*– allele in mothers may affect the outcome of children with autism. Clearly, it is crucial that careful assessments of behavioral components are included in all cohorts of families involved in genetic studies, to understand the role of genes, such as *DBH,* in susceptibility to or modification of the phenotype in individuals with autism.

Phentolamine N-Methyltransferase. The phentolamine *N*-methyltransferase (*PNMT*) gene is located at q21–q22 on chromosome 17. PNMT converts norepinephrine to epinephrine in the cytosol, and is taken up into vesicles for release. Two single nucleotide polymorphisms (SNP) in the promoter region, and one in intron 1 and the 5′ flanking region have been identified (Wu and Comings, 1999; Saito et al., 2001). PNMT was found to be associated with sporadic early-onset but not late-onset Alzheimer disease (Mann et al., 2001). To date, no studies on this gene have been carried out in relationship to autism, although we have initiated such studies.

GENES INVOLVED IN THE CATABOLISM OF DOPAMINE AND NOREPINEPHRINE

Monoamine Oxidase-A. The monoamine oxidase-A (*MAOA*) gene is located on the X-chromosome at p11.23–.4. MAOA is a major enzyme involved in the catabolism of catecholamines, thus mutations or variations that affect levels of this enzyme are expected to result in relatively higher or lower amounts of dopamine and norepinephrine. Both abnormal behaviors (aggression) and borderline retardation have been reported in a family with a point mutation in the *MAOA* gene (Brunner et al., 1993). In addition, MAOA deficient mice have been found

to show both enhanced fear conditioning and passive avoidance learning whereas Pavlovian eye blink conditioning is unaffected (Kim et al., 1997), suggesting a primary effect of the deficiency on emotional learning in these mice. Given the frequent occurrence of aggressive behaviors and apparent lack of emotional responses in some individuals with autism, this gene is considered a good candidate gene for either an etiological role in autism or in modifying the phenotype in affected individuals.

An upstream variable number tandem repeat region (uVNTR) within the *MAOA* promoter region has been identified that consists of a 30 base-pair repeat sequence consisting of 3, 3.5, 4, or 5 copies (Sabol et al., 1998). Expression studies indicate that the number of repeats is related to transcriptional activity of the gene, with the 3-repeat allele showing reduced transcription relative to the other alleles (Sabol et al., 1998; Denney et al., 1999). Schmidt et al. (2000) reported an association between the 3-repeat allele and antisocial alcoholism. The 3-repeat allele has also been associated with decreased aggressiveness/impulse control problems and increased serotonergic responsivity (prolactin response to fenfluramine challenge) (Manuck et al., 2000).

In a recent family-based and population study, Yirmiya et al. (2002) examined this uVNTR in 48 families with autism (33 simplex families and 15 MPX families) and did not find an association between autism and these *MAOA* alleles, although they did note a trend for IQ to be lower in those with four or more copies of the repeat in the MPX families. These results are difficult to interpret, because the sample was quite heterogeneous, with ages ranging from 25 months to 33.6 years, and IQ tests varying and including either the Bayley, Cattell, Kaufman, or the WISC-R. However, the results are intriguing.

We also examined the *MAOA* uVNTR in both sets of MPX families and found a trend toward an increased frequency of low-expressing alleles in mothers of autistic children compared to controls in the first set of families ($\chi^2 = 3.094$; $df = 1$; $p = 0.08$) and a trend toward an increased frequency of mothers either heterozygous or homozygous for low-expressing alleles in the second set of families ($\chi^2 = 3.196$; $df = 1$; $p = 0.07$) (Figure 21.5). Furthermore, within these families, children whose mothers had at least one low-expressing allele had higher mean verbal communication scores than did those children whose mothers had two high-expressing *MAOA* alleles (mean: 14.40 compared to 11.48; $F = 5.707$; $p = 0.02$). In addition, more children whose mothers had low-expressing alleles scored high on repetitive behaviors (98% versus 82%; $\chi^2 = 4.051$; $df = 1$; $p = 0.04$). Thus, for the MPX families, *MAOA* does appear to affect the phenotypic expression of autism in affected individuals.

In a separate study of 41 males under the age of 7 years from simplex families, we found that children with the low-activity allele had both lower IQ and more

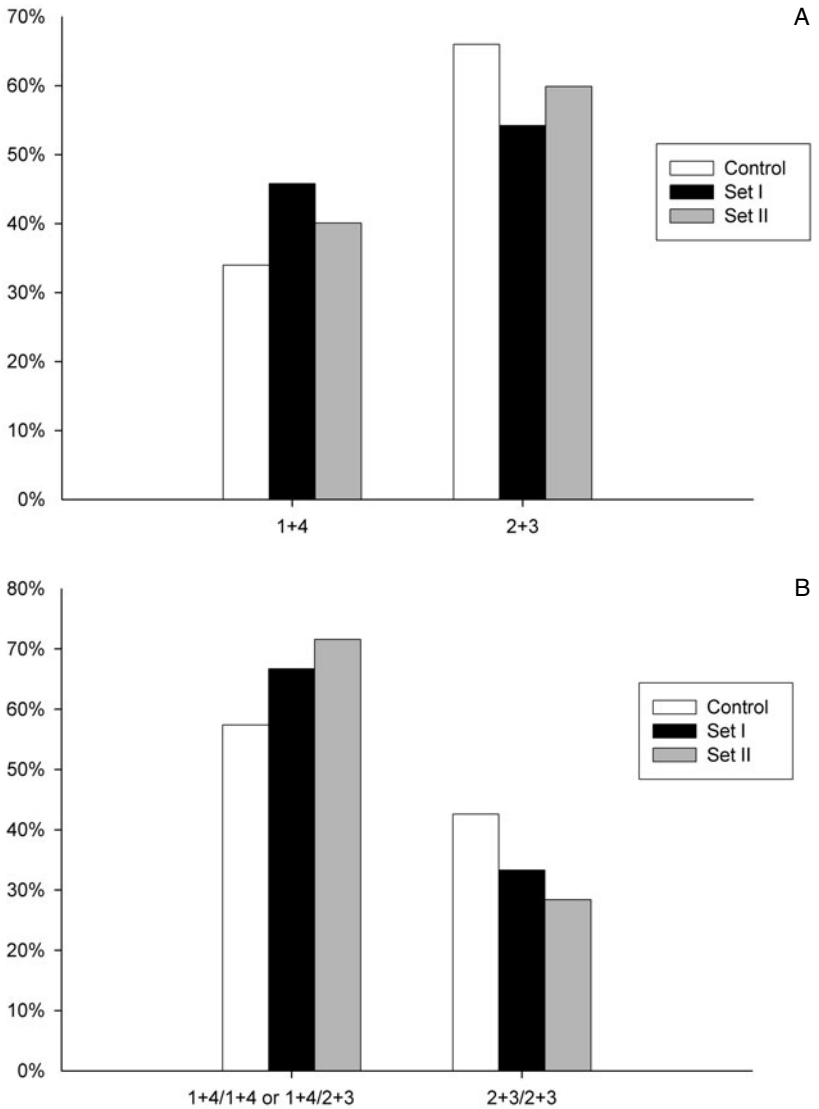

FIGURE 21.5. Comparisons of frequencies of *MAOA* uVNTR alleles in mothers from two cohorts of families with autism and a female comparison group. (A) Distribution of *MAOA*-uVNTR low (1, 4) and high-expressing (2, 3) alleles in mothers of autistic children and a female comparison group (Set I: $\chi^2 = 3.094$; $df = 1$; $p = 0.079$; Set II: $\chi^2 = 1.383$; $df = 1$; $p = 0.240$). (B) Distribution of *MAOA*-uVNTR genotypes containing at least one low-expressing allele versus homozygous high-expressing alleles in mothers of autistic children and a female comparison group ($\chi^2 = 0.578$; $df = 2$; $p = 0.447$; $\chi^2 = 3.196$; $df = 2$; $p = 0.074$).

severe autistic behaviors than did children with the high-activity allele. Children with the low-activity allele showed a worsening in IQ at 1-year follow-up, but no change in the severity of their autistic behavior (Cohen et al., 2003). Taken together, our findings on diverse groups of families with autistic children make *MAOA* a strong candidate gene for modifying the expression of autism, particularly in males.

Catecholamine-O-Methyltransferase. The catecholamine-o-methyltransferase (COMT) gene is located on chromosome 22 at q11.2. COMT is a major degradative enzyme for dopamine, norepinephrine, and epinephrine. It was shown that a val158met G→A transition leads to reduced heat stability and less activity (Lachman et al., 1996). Thus, this polymorphism in *COMT* has been studied in a large number of disorders. No association was found with Parkinson disease (Syvanen et al., 1997), schizophrenia (Daniels et al., 1996; Strous et al., 1997), major recurrent depression and bipolar disorder (Gutierrez et al., 1997; Syagailo et al., 2001), Ultradian rapid cycling in bipolar disorder (Geller and Cook, 2000), and attention deficit hyperactivity disorder (ADHD) (Hawi et al., 2000). However, the low-activity allele was more prevalent in late-onset alcoholism (Tiihonen et al., 1999), and the high-activity allele was more prevalent in major depressive disorder in females (Schulze et al., 2000) and in polysubstance abuse (Vandenbergh et al., 1997; Horowitz et al., 2000). We (Zhang et al., 2002) found an increased frequency of the high-activity allele in autistic children in both sets of families (Set I: $\chi^2 = 4.13$; $df = 1$; $p = 0.04$; Set II: $\chi^2 = 9.795$; $df = 1$; $p = 0.002$), and in all families combined ($\chi^2 = 12.27$; $df = 1$; $p = 0.001$) (Figure 21.6A). The increased frequency of the *val*-allele resulted from a higher frequency of *val/val* children than in the comparison group (*val/val* = 29.4%, *val/met* = 50.8%, *met/met* = 19.8% compared to 16.0%, 52.2% and 31.8%, respectively, in the comparison group; $\chi^2 = 13.51$; $df = 2$; $p = 0.001$) (Figure 21.6B). Increased *COMT-val* allele frequencies were also seen in both parents. Despite these clear allele and genotype frequency differences from controls, mean ADI-R scores did not differ for individuals with different genotypes in any of the three domains characterizing autism (i.e., reciprocal social interaction, verbal and nonverbal communication, stereotypic behaviors). However, there was a tendency for children with at least one *val* allele to have later age of first word ($\chi^2 = 3.71$; $df = 1$; $p = 0.05$). These results suggest that the *COMT* gene plays a role in modifying the phenotype in persons with autism.

GENES INVOLVED IN THE UPTAKE OF DOPAMINE AND NOREPINEPHRINE

In addition to genes affecting the synthesis and breakdown of catecholamines, the dopamine and norepinephrine transporters, as well as receptors, can affect

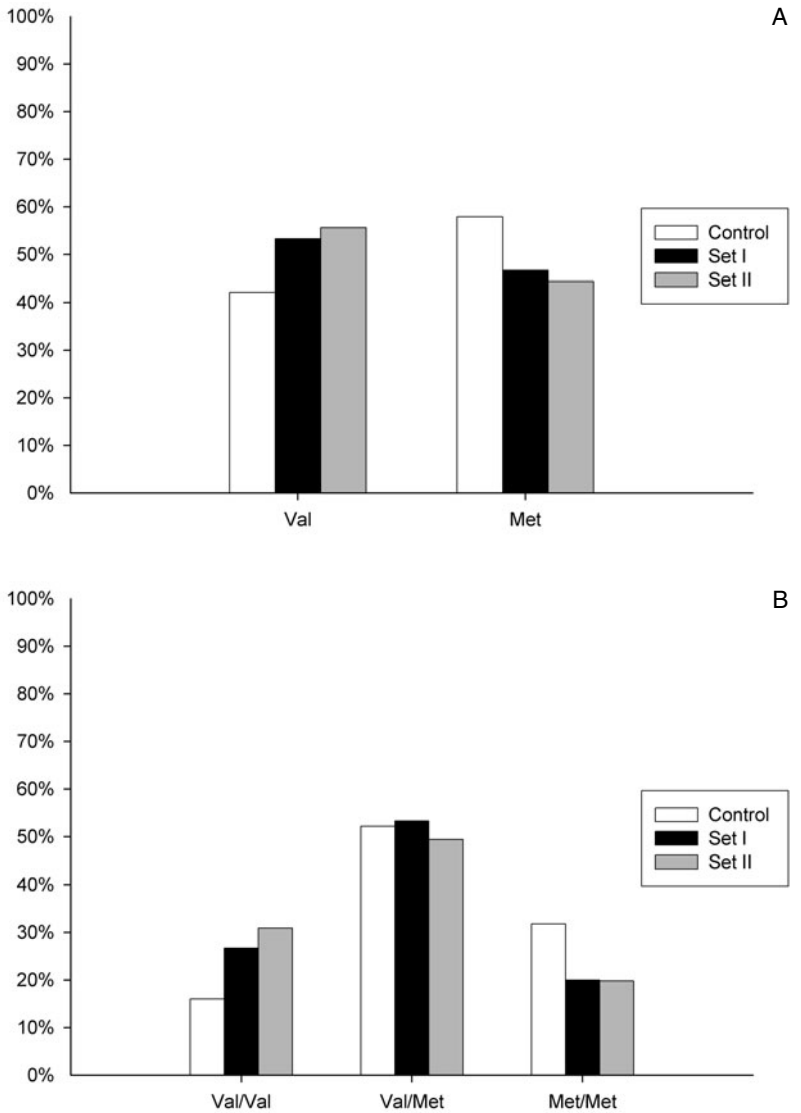

FIGURE 21.6. Comparisons of frequencies of *COMT* alleles in affected individuals from two cohorts of families with autism and a comparison group. (A) Distribution of *COMT-val* and *COMT-met* alleles in individuals with autism and a comparison group (Set I: $\chi^2 = 4.130$; $df = 1$; $p = 0.042$; Set II: $\chi^2 = 9.795$; $df = 1$; $p = 0.002$). (B) Distribution of *COMT val/met* genotypes in individuals with autism and a comparison group ($\chi^2 = 4.509$; $df = 2$; $p = 0.105$; $\chi^2 = 11.305$; $df = 2$; $p = 0.004$).

the effective level of dopamine and norepinephrine available at the synapse. Thus, these genes are worthy of consideration as candidate genes for ASD.

Dopamine Transporter and Norepinephrine Transporter. These transporter proteins terminate neurotransmission via the reuptake of neurotransmitters from the synapse into the presynaptic neuron for recycling or degradation. The transporter proteins are also expressed in a variety of other tissues, where they function to remove neurotransmitters from the bloodstream. The norepinephrine transporter, for example, is expressed in the placenta and is exposed to maternal blood (Balkovetz et al., 1989; Ramamoorthy et al., 1992), where its affinity for both dopamine and norepinephrine suggests a role for this protein in the transplacental transport of these neurotransmitters to the fetus. Altered transport, either increased or decreased, could affect exposure of the fetus to inappropriate neurotransmitter levels, resulting in abnormal development. Although dopamine transporter proteins are not present in the placenta, they could be involved in the response to maternal dopamine at the neuronal level in the developing fetus. Alterations in the function of one or more of these transporters could also result in abnormal synaptic transmission, leading to some autistic behaviors, or in the altered serum transmitter levels observed in autistic individuals and their relatives (Anderson, 1987). Thus, we are interested in determining whether there are specific variants in families with autistic children for these genes that make the transporter proteins more or less efficient or that regulate the numbers of these molecules present on the target membranes. The dopamine transporter gene (DAT) is located on chromosome 5 (5p15.3) and the norepinephrine transporter gene (NET) is on chromosome 16 (16q12.2). Using linked polymorphisms, we were able to show that both of these transporter genes appear to be important in susceptibility to autism (Polley et al., submitted). However, these results are based on linked polymorphisms, rather than intragenic markers (which were only recently discovered), and we are thus starting to examine intragenic SNPs to determine whether our findings further implicate the *DAT* and *NET* transporter genes or other linked genes in the etiology of ASD.

Dopamine Receptors. The five dopamine (DA) receptor genes (*DRD1:* 5q35.1; *DRD2:* 11q22–23; *DRD3:* 3q13.3; *DRD4:* 11p15.5; *DRD5:* 4p15.1–15.3) have been studied in a variety of populations, but few studies have been done on families with autistic children. Feng et al. (1998) examined the coding regions of the *DRD1* and *DRD5* genes in 25 autistic subjects using restriction endonuclease fingerprinting. They found no changes in the *DRD1* gene and a silent polymorphism in the *DRD5* gene, as well as a novel missense mutation in transmembrane domain II at a highly conserved amino acid in one subject with autism. As far as we are aware, there have been no other studies to determine whether this change is present in other persons with autism.

Clearly, few studies on the transporter and receptor genes have been undertaken in this population to date. The availability of intragenic polymorphisms (particularly in the *DAT* and *NET* genes) with possible functional effects will enable more detailed studies on these genes in the future.

Conclusion

The identification of genes for complex genetic disorders is a difficult task—especially when the disorder, like autism, is both etiologically and genetically heterogeneous and there are few obvious clues on how to assign families into more homogeneous groups. The inclusion of families with different etiologies will decrease the chance of identifying genes of small or even moderate effect. In the studies presented here, it is quite clear that the two sets of families—recruited in different ways—are genetically different. Several genes within the dopamine pathway show trends or significant findings in Set I families, but not in Set II families. Whether this represents chance findings or some unknown phenotypic differences between the two sets of families is difficult to resolve. The broad spectrum of phenotypes among individuals with ASD and their family members must be carefully considered and measured, so that families with similar "phenotypes"—be those physical or family history characteristics—can be grouped together for genetic analyses. The identification of subtle morphologic differences among affected individuals may provide clues to the timing of developmental insults and a means for grouping similarly affected families. For example, genetic studies on individuals with autism who have ear abnormalities may lead to more consistent findings, given that such individuals share a lesion that occurred at a specific time during embryogenesis. We and others are attempting to identify physical differences among individuals with autism, using three-dimensional imaging of facial features. Such studies are more likely to result in the identification of both etiologically important genes for autism and modifier genes that result in different behavioral phenotypes. An alternative approach is to group families according to genetic differences, and then attempt to identify additional genes that follow the same trends in the separation of the families. It may be necessary to use both approaches to tease out those genes that affect core aspects of the phenotype of ASD and those that exacerbate or ameliorate the symptoms. The challenge is to include as much information as possible in the characterization of families and to use this information creatively to help identify genes important in the susceptibility to ASD, with the eventual aim of understanding the pathophysiology and developing means for successful intervention and prevention of the debilitating aspects of this challenging disorder.

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22

Serotonin in Autism

George M. Anderson, Ph.D.

The initial isolation and identification of serotonin (5-hydroxytryptamine [5-HT]) was accomplished in the late 1930s through the early 1950s. Researchers demonstrated the presence of 5-HT in blood and in enterochromaffin cells of the gut and established the indole structure of the molecule. In 1953, a clam heart bioassay was used to show that mammalian brain contained substantial (on the order of picograms per gram) quantities of 5-HT. An appreciation of the actions of 5-HT as a mammalian vasoconstrictor and mollusc cardiostimulant, the parallels between 5-HT and the then newly discovered neurotransmitters norepinephrine and acetylcholine, and commonalities with the molecular structure of lysergic acid diethylamide (LSD) led to suggestions that 5-HT might play a role in mental disorders (for reviews, see Sjoerdsma and Palfreyman, 1990; Whitaker-Azmitia, 1999). In 1961, Schain and Freedman reported finding elevated levels of platelet 5-HT in individuals with autism; serious consideration of the possible role of 5-HT in autism dates from that time.

Although the platelet hyperserotonemia of autism has continued to provide a major impetus for the study of 5-HT in autism, a wide range of neurobiologic, pharmacologic, and genetic data has contributed to the continuing interest in this area. As background, the central serotonergic system is depicted in Figure 22.1. As shown, nearly all cell bodies of central 5-HT neurons are located in the dorsal midbrain and brainstem and project throughout the brain in a pervasive fashion. 5-HT is produced from the dietary amino acid precursor, tryptophan, and metabolized predominantly to 5-hydroxyindoleacetic acid. As might be expected from its phylogenetically ancient role in neural transmission and its extensive central nervous system (CNS) projections, 5-HT has been shown to play a key role in a range of behaviors and processes, including sensory gating and processing, behavioral inhibition, appetite, aggression, sleep, mood, affiliation, and neuroendocrine secretion.

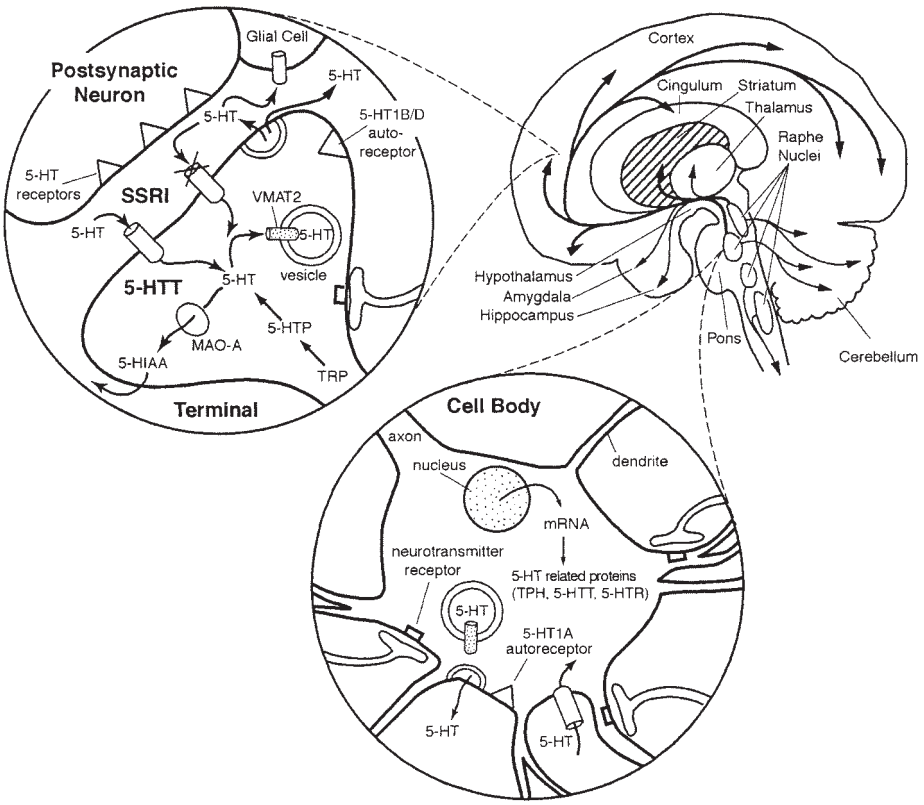

FIGURE 22.1. The central serotonergic system. Raphe nuclei of the brainstem contain the cell bodies of serotonergic neurons. Projecting rostrally to the brain and caudally to the spinal cord, serotonin (5-HT) is pervasive in the CNS. Especially rich innervation is present in the basal ganglia, amygdala, hippocampus, and hypothalamus. Serotonin-related proteins are produced after transcription of appropriate genes. The membrane 5-HT transporter (5-HTT), the less specific vesicular transporter (VMAT2, used for loading 5-HT into synaptic vesicles), the 5-HT1A autoreceptor, and the synthetic enzyme tryptophan hydroxylase (TPH) are found in the cell body. These proteins (with the exception of the 5-HT1A receptor) are also found in the terminal region, along with the catabolic enzyme monoamine oxidase A (MAO-A) and the 5-HT1B/D terminal autoreceptor. 5-HT is synthesized after hydroxylation of tryptophan (TRP) and decarboxylation of 5-hydroxytryptophan (5-HTP). Eventually most of the 5-HT produced is metabolized to 5-hydroxyindoleacetic acid (5-HIAA); however, extracellularly released 5-HT is mainly cleared by uptake through the 5-HTT. Blockade of the 5-HTT by selective serotonin reuptake inhibitors (SSRI) leads to increased extracellular and synaptic levels of 5-HT and increased stimulation of autoreceptors and postsynaptic receptors. Receptors for other neurotransmitters (heteroreceptors) and autoreceptors on the cell body and terminal region control neuronal firing rate and release of 5-HT.

Theoretical considerations underlying the rationale for the study of 5-HT in autism include the role of 5-HT in neurodevelopment, its rich innervation of limbic areas critical for emotional expression and social behavior, and the aforementioned involvement of 5-HT in a wide range of behaviors—those often observed to be affected in individuals with autism. Providing a more empirical basis are studies showing the therapeutic benefit of serotonergic agents in autism, reports of 5-HT-related neuroendocrine abnormalities, the well-characterized platelet hyperserotonemia, and reported associations between autism and 5-HT-related genes.

Serotonin and Neurodevelopment

The critical role of 5-HT in guiding neurodevelopment and the extended ontogeny of the central serotonergic system provide a theoretical basis for positing the possible involvement of 5-HT in the etiology and pathophysiology of autism. Serotonin and its associated transporter and receptors are found very early in development, appearing by E11–12 in the rat and by 5 weeks of gestation in humans (Rubenstein, 1998; Whitaker-Azmitia, 2001). Much of the early expression of 5-HT appears to be related to its role as a growth factor and regulator of neuronal development. Thus, in addition to functioning as a neurotransmitter and modulator of neural transmission in the more developed brain, 5-HT appears to have critical effects on neurogenesis, morphogenesis, and synaptogenesis in the developing brain (Whitaker-Azmitia and Azmitia, 1994; Moiseiwitsch and Lauder, 1995; Mazer et al., 1997). Serotonin genetics and neurodevelopment intersect in the recent observation of an effect of *HTT* (5-HT transporter) intron 2 variable number tandem repeat (VNTR) alleles (Stin2.10 and Stin2.12) on patterns of transporter gene expression in the developing mammalian brainstem (MacKenzie and Quinn, 1999). Accumulating evidence indicates that 5-HT projections continue to undergo age-related change through early adulthood and that the serotonergic system is particularly plastic.

Neuroanatomic Considerations

Although a number of brain areas have been suggested to play a role in the etiology and pathophysiology of autism (Waterhouse et al., 1996), a particularly strong rationale has been developed for involvement of the amygdala and associated areas of the limbic cortex. Thus, consideration of the fundamental socialization deficit in autism and of the behavioral sequelae of amygdalar

lesions (Bachevalier, 1994), as well as the results of cytologic analysis (Kemper and Bauman, 1998), have made these brain areas of particular interest. More specifically, the core social relatedness deficits in autism serve to focus attention on the rostral limbic system, including the amygdala, septum, medial orbitofrontal cortex, anterior insular cortex, anterior cingulate cortex, and the nucleus accumbens (Kemper and Bauman, 1993; Lotspeich and Ciaranello, 1993; Barbas and Blatt, 1995; Devinsky et al., 1995). Additional limbic-related areas of possible interest include the subiculum, hippocampus, parahippocampus cortex, and dorsolateral prefrontal cortex. The various limbic areas are richly enervated with serotonergic projections, and the nucleus accumbens, an area crucial in appetitive and reward processes, has an especially dense serotonergic innervation.

The cerebellum has also received intensive study in autism, with cerebellar alterations suggested due to its role in attention regulation and motor control (Pierce and Courchesne, 2001), and on the basis of results of postmortem (cytologic) and imaging studies. Researchers have consistently found neuropathologic abnormalities in the cerebella of individuals with autism (Bauman and Kemper, 1985, 1996; Ritvo et al., 1986; Waterhouse et al., 1996). Specifically, a reduced number of Purkinje neurons have been reported in autistic cerebellar hemispheres, with normal cell counts observed in vermal segments. The data from neuroimaging studies are less consistent, with vermal hypoplasia, hyperplasia, and unaltered vermal volumetrics having been reported in autism (Minshew et al., 1997). At present, the existence of gross volumetric abnormalities in the vermis and their possible relationship to the reduced Purkinje cell number in the hemispheres remain to be established. However, the neuropathologic findings, by themselves, prompt consideration of the possible role of the cerebellum in autistic behaviors. Although 5-HT innervation of the cerebellum is less prominent than in most other brain areas, 5-HT projections are critical for cerebellar function, and 5-HT appears to play a critical role in the formation of the cerebellar cortex. It is clear that the serotonergic input has an important role in modulating the activity of cerebellar neurons (Hokfelt and Fuxe, 1969; Bishop and Ho, 1985; Maura and Raiteri, 1996). Although the exact role of 5-HT in cerebellar development is not known, it appears that 5-HT1A receptors have an early and rich expression in the rodent and human cerebellum (Miquel et al., 1994; Slater et al., 1998). The presence of the 5-HT1A receptor in the adult human cerebellum has been established on the bases of binding studies (Hall et al., 1997), immunocytochemistry (Matthiessen et al., 1993), and pharmacologic modulation of in vivo 5-HT release (Mendlin et al., 1996).

The Role of Serotonin in Autism-Relevant Behaviors

In adult animals, 5-HT is seen to play intrinsic roles mediating the diverse, yet autism-relevant, behaviors of sleep, mood, arousal, aggression, impulsivity, and affiliation (Lucki, 1998; Barnes and Sharp, 1999). In summary, reduced serotonergic function has been associated with worsened sleep, depressed mood, altered arousal, increased aggression, greater impulsivity, and reduced social behavior. Although 5-HT tends to play an inhibitory role in the CNS, its actions are complex and depend greatly on the specific location and class of receptor stimulated. For instance, although markedly increasing extracellular 5-HT release can reduce appetite and aggression, it also can lead to a syndrome of distinctive repetitive behaviors. Genetic data from a range of disorder association studies have tended to connect 5-HT-related genes to disorders defined by symptoms in these areas of behavior, including mood, social phobia, and obsessive-compulsive and anxiety disorders (Veenstra-VanderWeele et al., 2000).

Serotonergic Drug Treatment and Challenge Studies

Drug treatment studies have demonstrated that agents affecting the 5-HT system can be useful for improving symptoms in individuals with autism. Drugs targeting the 5-HT transporter (including the 5-HT reuptake inhibitors, fluoxetine and fluvoxamine) are widely used in autism (Cook et al., 1992; Gordon et al., 1993; McDougle et al., 1996a; Martin et al., 2003). Risperidone, another frequently used medication (RUPP Autism Network, 2002), is an atypical neuroleptic that acts as a potent antagonist at the $5HT_{2A}$ receptor. In the most recent of the selective serotonin reuptake inhibitor (SSRI) studies, there was discussion of the factors that may critically affect SSRI treatment response and adverse effects in individuals with pervasive developmental disorders (PDD) (Martin et al., 2003). The pharmacokinetics and pharmacodynamics of serotonergic agents can be affected by drug metabolism, bioavailability, ontogeny of the 5-HT system, and gender. The genetic bases underlying differences in these areas (the pharmacogenetics) are just beginning to be examined.

Although the primary mechanism of action of SSRIs is competitive inhibition of the neuronal serotonin transporter protein, leading to elevated synaptic levels of serotonin, the presumed therapeutic alterations in central serotonergic neurotranmission must be mediated through increased stimulation of pre- and postsynaptic serotonin receptors. It is now clear that a number of ontogenic changes occur in the serotonin system. These include early changes in neuronal response to serotonin as a factor affecting neuronal growth, neuritogenesis, and

synaptogenesis (Lauder, 1995); an extended ontogeny in the decline of cerebrospinal levels of the serotonin metabolite 5-hydroxyindoleacetic acid (Riddle et al., 1986); ontogenic changes in serotonin receptor densities (Saxena, 1995); changes in platelet serotonin with puberty (McBride et al., 1998); and changes in serotonin-mediated neuroendocrine response (McBride et al., 1990). The data from these lines of evidence strongly point to a neurotransmitter system in flux throughout childhood and adolescence. In this situation, one would expect the pharmacodynamics of any serotonergic agent, such as the SSRIs, to change substantially with development.

Although developmental changes may be important, the extent of reuptake blockade achieved in a particular patient is a fundamental issue. This can be estimated indirectly by looking at the extent of serotonin reuptake blockade in the platelet. Nearly all (>99%) of whole blood serotonin is found in the platelet. This platelet serotonin is exogenously derived, being taken up via the platelet serotonin transporter over the approximate 10-day life-span of the platelet. The percentage decrease in platelet serotonin after treatment with an SSRI gives a time-averaged indication of the degree of transporter blockade. We and others have used this index to determine the mode of action of antidepressants (Narayan et al., 1998), to assess exposure to reuptake inhibitors in nursing infants and mothers treated for postpartum depression (Epperson et al., 2001), and to estimate the extent of reuptake blockade in children with PDD treated with fluvoxamine (Martin et al., 2003).

In our recent low-dose fluvoxamine treatment study (N = 18; maximum dosage: 1.5 mg/kg/day), drug efficacy and tolerability were assessed in youngsters with PDDs accompanied by anxiety symptoms or obsessive-compulsive symptoms. Although there were no significant benefits for the group as a whole, eight subjects (including all four females) showed a positive response (Martin et al., 2003). Despite the lower dose level, measurement of serotonin reuptake inhibition in the platelet showed 80–95 percent blockade in responders and nonresponders alike, suggesting that response and adverse effects were probably not due to either under- or overmedication. The excellent response seen in females warrants careful follow-up, as gender-based differences may be very important in determining clinical response and would have important clinical and theoretical implications. There are two recent reports showing substantially higher female versus male response rates in adult patients treated with SSRIs for depression (Kornstein et al., 2000) and in individuals with posttraumatic stress disorder (Davidson et al., 2001).

The field of pharmacogenetics is in its infancy; however a surprising amount of relevant data are available regarding the influence of serotonin-related gene variants on the clinical response to serotonergic agents. There are a number of

intriguing studies examining the role of serotonin receptor gene polymorphisms in atypical neuroleptic response (Masellis et al., 2000), and a good deal of attention has been focused on a biallelic insertion/deletion promoter polymorphism (5-HTTLPR) of the serotonin transporter gene (Anderson and Cook, 2000; Veenstra-VanderWeele et al., 2000). Initial studies in depression have provided evidence that the promoter polymorphism influences response rate and latency to response in depressed individuals treated with SSRIs (Smeraldi et al, 1998). In a recent report, the prevalence of SSRI-induced mania was much greater in depressed patients with the deletion (short allele) allele (Mundo et al., 2001). Thus, the 5-HTTLPR genotype may be an important predictor of beneficial and adverse effects of SSRI treatment and may be of particular relevance in young PDD patients given the behavioral activation that is often seen in such subjects.

Paralleling the treatment studies are neuroendocrine and behavioral challenge paradigms employing serotonergic agents. These studies have found abnormal responses to the 5-HT releaser fenfluramine (McBride et al., 1989), the 5-HT precursor 5-hydroxytryptophan (Y. Hoshino, personal communication, 1991) and the 5-HT_{1B} receptor agonist sumatriptan (Novotny et al., 2000). The fenfluramine and 5-HTP studies both observed a lowering or blunting of the prolactin response, whereas sumatriptan produced an elevated response in autism. The data are not definitive, but they are consistent with decreased central presynaptic 5-HT functioning in autism. Depletion of the 5-HT precursor tryptophan resulted in a significant increase in autistic behaviors (McDougle et al., 1996b).

Serotonin-Related Genes and Expression of Autism

Family and twin studies clearly show that autistic behavior is largely genetically determined and suggest a heterogeneous and polygenetic basis. Molecular genetic studies indicate that, in most affected individuals, a large number (>10) of genetic variants are contributing to the altered behavior. Interestingly, it also appears that the variants involved are common polymorphisms. It is natural to suggest that different groups of variants are involved in affecting different components of altered behavior. It also seems likely that certain genes and their variants may play a role across domains of behavior; 5-HT-related genes are good candidates for exerting such epistatic and pleiotropic effects. It is unclear whether the same variants confer risk to disordered behavior across syndromes, whether the behaviors are continuous with normal behaviors, and whether genetic and behavioral interactions across domains are critical for the expression of full-blown autism (Cook, 2001; Lauritsen and Ewald, 2001).

Genes encoding a number of the components involved in 5-HT neural transmission (see Figure 22.1) have been considered as possible contributors to behav-

iors and disorders of potential relevance to autism (Anderson and Cook, 2000; Veenstra-VanderWeele et al., 2000). Research in autism per se has examined 5-HT2A variants (Veenstra-VanderWeele et al., 2002), but has largely focused on the influence of the 5-HT transporter gene (*HTT*). The *HTT* locus SLC6A4 (found on chromosome 17, region q11.1–q12) is a leading candidate gene, given the nature of the biologic findings and pharmacologic effects observed in autism. Of special interest is the biallelic promoter region polymorphism 5-HTTLPR. The promoter variant consists of a 44 base pair deletion/insertion in a repeat region of the promoter. Over the past several years, there have been reports of preferential transmission of the *s* allele (Cook et al., 1997; Kim et al., 2002), preferential transmission of the *l* allele (Klauck et al., 1997; Tordjman et al., 2001; Yirmiya et al., 2001), and an absence of preferential transmission in individuals with autistic disorder (Maestrini et al., 1999; Persico et al., 2000) (Table 22.1). Although, taken together, the studies do not convincingly support a role for *HTT* variants in determining risk, one of the latter studies did observe an as yet to be replicated allelic association with severity of communication and social interaction impairment (Tordjman et al., 2001). Further work is required to establish the possible modifying effect of the polymorphism; careful measurement of domain impairment and new approaches to studying possible modifying loci are recommended. The potential importance of looking at the genetic bases for components of autistic behavior is underlined by recent research findings that the promoter polymorphism may influence the response of the amygdala to fearful faces (Hairi et al., 2002), the acquisition of fear conditioning (Garpenstrand et al., 2001), and infant orientation to social stimuli (Ebstein et al., 1998).

Neurochemical Alterations

Although most of the 5-HT-related neurochemical research has focused on the hyperserotonemia of autism, a number of studies of cerebrospinal fluid (CSF) 5-HIAA (5-hydroxyindolacetic acid) and several neuroendocrinologic studies of central 5-HT functioning have been reported (Anderson, 1987, 1994; Cook, 1990; Anderson and Hoshino, 1997). CSF studies are in general agreement that little or no difference exists between autistic and control groups' mean levels of the major 5-HT metabolite 5-HIAA (Narayan et al., 1993).

The basic finding of elevated platelet 5-HT is robust and well replicated (Anderson et al., 1987, 1990; Cook and Leventhal, 1996). Increased levels are observed regardless of whether concentrations are expressed as amount per volume of blood (e.g., nanograms per milliliter) or as amount per number of platelets (nanograms per 10^9 platelets); platelet counts and size distribution have been reported to be normal in autism. Although the basic finding of a group

TABLE 22.1. The Serotonin Transporter Promoter Polymorphism in Autism

Study Population	*Finding*	*Reference*
TDT analyses[a]		
North American	*s* allele preference: 48/77; $p = .030$	Cook et al. (1997)
German	*l* allele preference: 40/63; $p = .032$	Klauck et al. (1997)
North American/ European	No preference: *l* allele 76/148	Maestrini et al. (1999)
Israeli	*l* allele preference: 25/36; $p = .025$	Yirmiya et al. (1999)
Italian/North American	No preference: s allele 48/84	Persico et al. (2000)
French	*l* allele preference:[b] 40/64; $p = .046$	Tordjman et. al. (2001)
North American	*s* allele preference:[c] 72/115; $p = .007$	Kim et al. (2002)
Sib-pair studies (genome screens)		
North American/ European	No increased sharing in 17q11.1–q12 region	IMGSAC (1998)
North American/ Australian	No increased sharing in 17q11.1–q12 region	Risch et al. (1999)
Allele frequency study		
North American	*s* and *l* allele frequencies similar to control	Zhong et al. (1999)

[a] Transmission disequilibrium test: the expected rate of transmission for a particular allele is 50%, or one-half of the possible transmissions.
[b] No preference when compared to unaffected siblings (40/64 versus 25/44; $p = 0.55$).
[c] Included 37 trios from Cook et al. (1997). Of note, several other *HTT* polymorphisms showed greater preferential transmission and strong linkage disequilibrium was observed in the vicinity of *HTT*.

mean increase in autistic subjects is well established, the magnitude of the increase is not clear. Recent research has indicated that substantial racial and pubertal effects exist; when these were accounted for, a group mean increase of 25 percent was observed (McBride et al., 1998). The relationship of blood 5-HT to the behavioral aspects of autism and the distribution of the measure in the autistic group are also not clear. Serotonin levels have not been consistently correlated with degree of mental retardation (MR) or other symptomatology, and recent studies have not found an increase in the MR group. In groups of unmedicated autistic individuals, the distribution of blood 5-HT levels appears Gaussian,

suggesting that the group mean increase was not due to a subgroup of hyperserotonemic individuals. However, the possibility of a multimodal distribution cannot be ruled out, given the size of the groups examined.

The typical group mean increase observed in 5-HT is relatively robust; however, the high degree of overlap within the normal population has prevented the use of blood 5-HT levels in screening or diagnosis of autism. Numerous investigators have been intrigued, however, by the possibility of discovering the cause(s) of the elevation (Hanley et al., 1977; Young et al., 1982; Yuwiler et al., 1987; Anderson et al., 1990; Cook, 1990). It has been hoped that understanding the mechanism of hyperserotonemia would lead to the development of more specific and useful markers, elucidation of the CNS abnormality, and useful approaches to the treatment of the condition.

Two main possibilities have been considered as potential causes of the hyperserotonemia: (1) increased exposure of the platelets to free 5-HT as a result of either decreased catabolism or increased synthesis of 5-HT, and (2) altered platelet handling of 5-HT. The issue of decreased catabolism of 5-HT has been examined by measurements of platelet monoamine oxidase (MAO) activity and by measuring monoamine substrates and metabolites. These studies have strongly suggested that MAO activity, and hence 5-HT catabolism, is not altered in autism (Anderson et al., 1990). The normal excretion rates observed for 5-HIAA in autistic subjects in most, but not all, studies also strongly indicate that 5-HT synthesis is not increased in autism. Although these data strongly suggest that 5-HT metabolism is unaltered in autism, they do not definitely indicate whether the circulating platelet is exposed to increased levels of 5-HT. This issue can be addressed most directly by determining plasma free levels of 5-HT. When high-performance liquid chromatography (HPLC) was used to determine platelet-poor plasma 5-HT levels in autistic subjects, the levels were similar to those seen in normal controls (Cook et al., 1988). Urinary excretion rates for 5-HT in autistic subjects have also been measured, and the group mean was found to be similar to or slightly lower than that seen for normal subjects (Anderson et al., 1989). These measures of plasma and urine 5-HT levels, as well as the evidence regarding 5-HT metabolism, indicate that the platelet is not exposed to increased concentrations of 5-HT. This, in turn, strongly implies that the platelet's handling of 5-HT is altered in autism.

Accumulated evidence indicates that further research on platelet uptake, storage, and release of 5-HT is warranted. Despite work in a number of laboratories, no consensus has been reached concerning relative rates of 5-HT uptake by platelets from autistic and normal subjects (Anderson et al., 1990; Cook et al., 1993). The studies have been more consistent in not finding differences in the affinities for uptake in the two groups. Group differences also were not seen

when researchers examined the number (B_{max}) and affinity (K_d) of 5-HT transporter binding sites in autistic and normal individuals (Anderson et al., 1984; Weizman et al., 1987). The question of altered efflux has been studied in some detail after an initial report indicated that platelets from autistic subjects have a greater efflux of preloaded tritiated 5-HT during in vitro incubation. In a multicenter study, the consensus was that autistic and normal groups had similar mean efflux rates (Boullin et al., 1982). The 5-HT_2 receptor-mediated augmentation of adenosine diphosphate (ADP)-induced platelet aggregation has been found to be lower in autism (McBride et al., 1989). In addition to the blunted platelet augmentation response, the autistic group had lower numbers (B_{max}) of 5-HT_2 receptors detected by iodinated-LSD binding.

Recent investigations examining the possible role of *HTT* in the expression of autism have stimulated renewed interest in the role of the transporter in the hyperserotonemia. At this point, the *HTT* promoter polymorphism seems unlikely to contribute substantially to the hyperserotonemia of autism, given the allele frequencies and functional effects observed (see Table 22.1). To expand on this point, substantial allelic effects are seen on the rates of platelet 5-HT uptake, with the *l*/*l* genotype having approximately double the uptake seen in the *l*/*s* and *s*/*s* groups. However, there is only a slight, if any, excess of *l* alleles in the autism group, and the uptake rate explains less than half the variance in 5-HT level. Thus, only a very small proportion (if any) of the group mean elevation in platelet 5-HT levels can be explained on the basis of transporter promoter genotype (Anderson et al., 2002). Although the promoter polymorphism probably does not play a part in the elevation, the possible role of the transporter and aspects of its regulation (Anderson and Horne, 1992; Ramamoorthy and Blakely, 1999) cannot be ruled out. Recent research indicates that whole blood or platelet 5-HT levels are highly heritable (Ober et al., 2001; G. M. Anderson, unpublished data); identifying the genes of major effect may have important implications with respect to the hyperserotonemia of autism.

Future Directions

Although continued advances in the genetics of autism are expected, the major revelation of the past 10 years has been the daunting complexity of autism genetics. Improved drug treatment is likely using more specific agents and with the application of pharmacogenetics; however, inferences regarding etiopathophysiology based on drug effects will be tenuous. Early screening and the application of neuropsychology to the identification of quantitative behavioral phenotypes (e.g., eye tracking studies) will offer new perspectives for dissecting and understanding autism-related behavior.

At present, the general areas of postmortem research and neuroimaging seem to offer the greatest potential for rapidly advancing the field. The recent availability of postmortem brain tissue has opened a whole new realm of opportunity. Neurochemical and cytologic investigations of the autistic brain should no longer lag behind those in other areas of neuropsychiatry. The analysis of pre- and postsynaptic serotonergic measures across a range of cortical and subcortical regions should be particularly informative. A number of structural imaging studies, initial functional magnetic resonance imaging (fMRI) studies, as well as the limited imaging research examining central 5-HT functioning, indicate that continued work in this area will prove fruitful. Reciprocal interchange between imaging, neuropsychological, and postmortem research should be especially useful and illuminating. Finally, work on the bases of the platelet hyperserotonemia may provide critically important information regarding possible central 5-HT dysfunction; the advantages of having identified a specific biochemical alteration in a delineated cell type might be best exploited by applying gene array or expression technology to this question.

Determining neurochemical phenotypes may make critical contributions to pulling apart disorders. As has been pointed out with respect to schizophrenia, "Instead of . . . mapping [the disorder] per se, it may be fruitful to map the genetic basis for atypical biochemical or physiologic responses found in [the disorder]" (Lander, 1988:106). In fact, there should be occasions where biochemistry or neurochemistry can become the independent variable, creating meaningful subcategories for fruitful exploration in a fashion analogous to that seen in other, more biologically based fields of medicine (McBride et al., 1996; Heninger, 1999).

The autism phenotype is gradually becoming less mysterious, and the problems and research issues better defined. However, the complex and enigmatic nature of autism-related behaviors and their underlying determinants present a pressing and difficult challenge to neuroscience.

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23

The GABAergic System in Autism

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There is a paucity of information concerning the role of the gamma-aminobutyric acid (GABA)-ergic system in autism. This chapter therefore focuses on the possible role of abnormalities in the GABAergic system during development, as well as its alterations in the mature autistic brain.

Anatomy of the GABAergic System

GABA-A receptor subunits, along with synthesizing enzymes GAD65 and GAD67 (glutamic acid decarboxylases), and GABA appear very early in development and emerge at the same time that neurogenesis is evidenced. At this time, embryonic day 13 in the rat, the central nervous system (CNS) is primarily composed of a proliferating neuroepithelium (Barker et al., 1998, 2000). In lower mammals, there is then a widespread proliferation of GABAergic neurons during CNS neurogenesis that recedes in the perinatal period, a time when the postmitotic neurons are beginning to differentiate and to form functional circuits and networks (Schaffner et al., 1993). In the early stages of development, GABA may have a dual action, with a depolarizing effect on some GABA-A receptors, acting as an excitatory neurotransmitter, and an inhibitory effect on other immature neurons (Ben-Ari et al., 1997). The factors responsible for shifting from an excitatory effect at early stages of development to the inhibitory action of GABA at later stages of development are not known. However, the GABA-A receptor subunits appear at different stages of development, suggesting specific roles for different types. For example, $\alpha 4$, $\beta 1$, and $\gamma 1$ subunits are transiently expressed by neurons during their migration and in the perinatal period during radial migration of glial progenitors (Ma and Barker, 1995; Barker et al., 2000). In contrast, other GABA-A receptor subunits ($\alpha 3$, $\beta 3$, and $\gamma 2$) are expressed in late embryonic development and may be important for neuronal differentiation and circuit formation (Maric et al., 1997; Barker et al., 2000).

Responses to GABA depend on at least two classes of GABA receptors: inotropic GABA-A receptors and metabotropic GABA-B receptors (for a review, see Martin and Tobin, 2000), with GABA-A receptors mediating most of the rapid inhibitory synaptic transmission in the CNS (Olsen and Homanics, 2000). On the Purkinje cells, GABA-A receptors are on the soma and dendrites, and GABA-B receptors are on their dendrites, with the latter therefore densely represented in the molecular layer (Billard et al., 1993; Vigot and Batini, 1997). Meinecke et al. (1989) reported that in rat and monkey cerebellum, the other four types of cerebellar cortical neurons (basket, stellate, Golgi type II, and granule cells) contain GABA-A receptors, and Turgeon and Albin (1993) noted GABA-B receptors on basket and stellate cells. Both Kato and Fukuda (1985) and Vigot and Batini (1997) also suggested that GABA-B receptors are located presynaptically on olivocerebellar climbing fibers, and Vigot and Batini (1997) reported postsynaptic GABA-B receptors on parallel fibers.

Both GABA-A and GABA-B receptors are found on neurons in the cerebellar nuclei, but only GABA-A receptors appear to be activated by Purkinje cells (Morishita and Sastry, 1995; Sastry et al. 1997). The neurons in the cerebellar nuclei provide a direct GABAergic nucleo-olivary feedback to the same neurons in the inferior olive that innervated them with climbing fiber collaterals (De Zeeuw et al., 1994, 1997). Application of GABA receptor antagonists to the inferior olive or lesioning of parts of the cerebellar nuclei suggest that GABAergic cerebellar terminals from the cerebellar nuclei are involved in the regulation of electrotonic coupling in the inferior olive and dynamically reassemble functional olivary networks (de Zeeuw et al., 1998).

Neuropathologic Changes Implicating the GABAergic System

Neuropathologic studies of the brains of autistic individuals have emphasized consistent abnormalities in the cerebellar system and the limbic system (Bauman and Kemper, 1994; Kemper and Bauman, 1998), with many of the pathologic changes implicating the GABAergic system. One of these is a statistically significant decrease in the number of the GABAergic Purkinje cells in the cerebellum (Ritvo et al., 1986; Arin et al., 1991), with up to an 80 percent decrease in the posterolateral neocerebellar and adjacent archicerebellar cortex and relative sparing of the vermis (Arin et al., 1991). The Purkinje cells project to the largely GABAergic cerebellar nuclei, which also have shown neuropathologic abnormalities. The neurons of the globose, emboliform, and fastigial cerebellar nuclei were found to be enlarged and plentiful in all of the autistic cases less than 12 years of age, but were observed to be small, pale, and decreased in number in

all subjects older than 22 years (Kemper and Bauman, 1998). The cells of the dentate nucleus were enlarged and plentiful in the young autistic patients, but did not show the later atrophy and reduced neuronal number with age that characterized the other cerebellar nuclei (Kemper and Bauman, 1998). The inferior olivary neurons in the brainstem, which project to the Purkinje cells, also show abnormalities. These cells were small and pale in all of the autistic brains older than 22 years of age, whereas those of the younger autistic brains were enlarged. Some cases also show ectopic neurons, presumed to be inferior olivary and/or arcuate neurons (Bailey et al., 1998; T. Kemper, personal communication).

In the limbic system, a consistent neuropathologic finding has been smaller neurons and an increased packing density in the hippocampal formation, entorhinal cortex, and amygdala (Bauman and Kemper, 1985, 1994), suggesting a curtailment of normal development. In agreement with this interpretation is a study of individual Golgi stained pyramidal cells from the CA1 and CA4 hippocampal fields, showing a reduced complexity and extent of their dendritic arbors in the autistic brain (Raymond et al., 1996). Although the etiology of this neuropathologic change has not been elucidated, an intriguing possibility is an early perturbation in the GABAergic system at a time when GABA normally has an excitatory role in neuronal development. In this regard, Shao et al. (2003) recently reported a strong linkage with the gene for the GABARβ3 receptor in autistic individuals, a GABA-A receptor subunit that is expressed at early stages of neuronal development (Maric et al., 1997; Barker et al., 2000). Further evidence for involvement of the GABAergic system in the limbic system has been provided by Blatt et al. (2001). In this survey, eight types of neurotransmitter receptors from four different systems were studied in the hippocampal formation. These included the serotonergic (^{3}[H]-8OH-DPAT-labeled 5-hydroxytryptamine [5-HT]$_{1A}$ receptors and ^{3}[H]-ketanserin-labeled 5-HT$_2$ receptors), cholinergic (^{3}[H]-pirenzepine-labeled M1 receptors and ^{3}[H]-hemicholinium-labeled high affinity choline uptake sites), glutamatergic (^{3}[H]-MK801-labeled *N*-methyl-D-aspartate [NMDA] receptors and ^{3}[H]-kainate-labeled kainate receptors), and GABAergic receptors (^{3}[H]-flunitrazepam-labeled benzodiazepine binding sites and ^{3}[H]-muscimol labeled GABA-A receptors). Only the two GABAergic receptor markers demonstrated a statistically significant reduction, a reduction that was found in the normally high binding regions (for ^{3}[H]-flunitrazepam: in the stratum pyramidale of CA2, prosubiculum and subiculum and stratum moleculare of the subiculum and for ^{3}[H]-muscimol: in the stratum pyramidale of CA1). A subsequent multiple concentration saturation binding experiment further demonstrated a trend for significance that the number of ^{3}[H]-flunitrazepam-labeled benzodiazepine binding sites were reduced in 10 of 11 hippocampal laminae

quantified (decreased B_{max}), whereas the binding affinity for the ligand (K_d) was normal in all 11 laminae (Blatt et al., 2002).

Possible Effect of Decreased Number of Purkinje Cells on the Cerebellar Circuitry

The major decrease in the number of Purkinje cells in the autistic cerebellum directly indicates that the GABAergic system is perturbed. To what extent the intrinsic and extrinsic cerebellar pathways may be altered and/or whether compensatory mechanisms are operative has not been established. One can only assume that, because cellular changes are found in the olivocortical-nuclear-olivo circuit in autistic individuals, some type of reorganization of their anatomic interrelationships occurs.

Because Purkinje cells provide the main targets for olivocerebellar climbing fibers, how is the olivocerebellar projection affected in autism? The possibilities include:

1. There is a secondary (retrograde) loss of inferior olivary neurons and thus, their projections to the missing Purkinje cells are absent;
2. The olivary cells survive and are sustained by their collaterals to the cerebellar nuclei;
3. The climbing fibers destined for their missing targets form heterologous synapses on granule cells or another cell type; and
4. The climbing fibers destined for their missing targets seek out alternate Purkinje cell targets within the same cerebellar region, forming supernumerary synapses on the remaining Purkinje cells.

The first possibility is unlikely, because observations of the lateral part of the principal olive, the part that is related to the atrophic posterolateral cerebellar cortex, showed no retrograde neuronal loss (Bauman and Kemper, 1985). The principal olive, however, does show age-related changes, with the lateral principal olive showing hypertrophied neurons in young cases and abnormally small, pale neurons in older cases. These observations led Bauman and Kemper (1994) to hypothesize that the Purkinje cell decrease must have occurred early in development.

The second possibility is that collaterals to the cerebellar nuclei sustain olive cells. However, in animal models of Purkinje cell death, only a small percentage of inferior olivary cells are maintained (Blatt and Eisenman, 1985a, 1985b, 1988, 1989, 1993). Thus, the hypothesis that collaterals of olivocerebellar fibers are

functioning through the cerebellar nuclei as a retention of "fetal circuitry," as proposed by Bauman and Kemper (1994), is possible, but it is a mystery as to why more inferior olivary cells are not lost secondary to the Purkinje cell reduction.

The third possibility—that heterologous synapses may occur in autism—is supported by observations in spontaneous cerebellar mouse mutants. In weaver mutant mice (wv/wv), in the absence of some Purkinje cells, olivocerebellar fibers distribute to the same cerebellar region but make aberrant contacts with granule cells. In Reeler mice (rl/rl), mossy fibers make heterologous synapses on Purkinje cells both in their normal position and when they are in ectopic positions (Wilson et al., 1981).

Finally, the fourth possibility—that some olivocerebellar climbing fibers hyperinnervate the remaining Purkinje cells—is a viable option. Physiologically, multiple innervation of Purkinje cells by climbing fibers (i.e., three or four climbing fiber inputs per Purkinje cell) has been demonstrated in a variety of genetic mutations in adult mice that have decreased numbers of Purkinje cells (e.g., Reeler, weaver, staggerer [sg/sg] mice) instead of the one climbing fiber per Purkinje cell in the normal adult (Crepel et al., 1980; Mariani and Changeux, 1980; Mariani, 1982). If the Purkinje cell decrease occurs prenatally, the normal developmental innervation of supernumerary climbing fiber inputs to Purkinje cells in autistic individuals might be maintained instead of the normal regression of inputs with a pairing back to the 1:1 climbing fiber:Purkinje cell ratio (e.g., Mariani, 1982).

In normal development, GABAergic basket cells form axonal perisomatic complexes or "nests" around Purkinje cell soma, providing a strong direct "disinhibition" of Purkinje cell output. An unanswered question in autism is whether some basket cells die off due to their decreased targets or are maintained by their synapses with the remaining Purkinje cells. If the Purkinje cell loss is prenatal, basket cell nests would not likely be present where their targets are absent. However, if there is a postnatal loss of Purkinje cells, it is likely that there will be "empty nests" present, where presumably a once-viable synapse had occurred. In the human disorder, in which there is a loss of Purkinje cells, silver staining shows empty Purkinje cell baskets as an index of the Purkinje cell loss (Graham and Lantos, 1997).

Because Purkinje cells are vastly reduced in number in the autistic brain, it is also likely that the amount of GABA in the cerebellum and/or its precursors may also be reduced. Consistent with this, Fatemi et al. (2002) reported a 50–51 percent decrease in the GABA-synthesizing enzymes GAD65 and GAD67 from different parts of the cerebellum. However, because these two rate-limiting enzymes for GABA are also found in other cerebellar GABAergic neurons, it is not clear whether the reductions reflect only a decreased Purkinje cell population.

Implications for a Perturbed Hippocampal GABAergic System

Much attention has been focused on GABAergic interneurons in the limbic system, especially in the hippocampal formation. These inhibitory interneurons play a critical role in regulating the complex interactions among principal cells (pyramidal and granule cells), and are thought to be intricately involved in memory-related plasticity (Freund and Buzsaki, 1996). In lower mammals, input from brainstem nuclei has been shown to terminate on specific types of GABAergic interneurons in the hippocampus. Septohippocampal neurons terminate via symmetrical synapses on parvalbumin-, calbindin-, and/or calretinin-positive hippocampal GABAergic neurons and on various peptidergic hippocampal interneuron subpopulations (Freund and Antal, 1988; Galyas et al., 1990; Miettinen and Freund, 1992a, 1992b; Acsady et al., 1993). GABAergic septohippocampal inputs terminate in the perisomatic region of parvalbumin-positive hippocampal interneurons, which, in turn, provide feedback inhibition to the perisomatic region of hippocampal principal cells, probably mediated by GABA-A receptors (Freund and Buzsaki, 1996).

Subsets of neurons from subpopulations of hippocampal GABAergic interneurons, including calretinin- and calbindin-positive neurons, selectively innervate other GABA interneurons and usually also colocalize with particular neuropeptides, such as vasoactive intestinal peptide or cholecystokinin (Acsady et al., 1996; Freund and Buzsaki, 1996; Galyas et al., 1996; Freund and Galyas, 1997). Such input may produce a rhythmic suppression or modulation of interneuron discharge (Freund and Galyas, 1997). This could result in synchronizing inhibitory cells that converge on groups of pyramidal cells, or alternatively, might disinhibit pyramidal cell dendrites targeted by particular excitatory inputs (Freund and Buzsaki, 1996).

If alterations in the extrinsic or intrinsic hippocampal GABAergic system in autistic individuals do result in altered function, then hippocampal efferents to key limbic cortical areas may also be affected. For example, recent studies in rhesus monkeys have revealed direct projections from the CA, prosubicular, and subicular subfields to the cingulate gyrus (Blatt and Rosene, 1989); medial and orbital frontal cortex (Barbas and Blatt, 1995); and to the posterior parahippocampal gyrus (Blatt and Rosene, 1998). This suggests that although obvious abnormalities in the cerebral cortex have not been noted in autistic brains via conventional Nissl stains, there may be a miswiring of connectivity between subcortical and cortical structures and/or functional alterations in the modulation of such connectivity via the GABAergic and/or other key neurotransmitter systems in the autistic brain. Interestingly, the "output" regions of the hippocampus may be most affected in autism, based on initial studies of GABA receptors. The most pronounced reduc-

tions in benzodiazepine binding sites and in GABA-A receptor density in adult autistic individuals were localized to high-binding regions in the hippocampus, including stratum pyramidale of CA1, prosubiculum, and subiculum, which are three hippocampal laminae described above as playing a critical role in efferent projections from the hippocampus to the cerebral cortex (Blatt et al., 2001).

Individuals with autism experience a proportionately high prevalence of seizures, with temporal lobe epilepsy developing in one-fifth to one-third of autistic individuals (Rutter, 1970; Gillberg et al., 1987; Olsson et al., 1988; Volkmar and Nelson, 1990; Goode et al., 1994; Bailey et al., 1998), suggesting an abnormality in the GABA system. In a positron emission tomography imaging study, the binding of ^{11}[C]-flumazenil to benzodiazepine sites in epileptogenic foci was found to be reduced with no change in binding affinity (Savic et al., 1988). Such changes in benzodiazepine binding are thought to reflect localized neuronal damage or neuronal loss in the regions with epileptogenic activity (Mohler et al., 2000). This must be taken into account when patients with a history of epileptic seizures are included in any imaging or in vitro studies measuring benzodiazepine/GABA-A ligand binding.

Another interesting development in autism research with possible implications to the GABAergic system is found in the results from the South Carolina Autism Project (Schroer et al., 1998), which has looked at chromosomal abnormalities in autism. Among the first 100 cases enrolled in this project, abnormalities of chromosome 15q11–13 have emerged as the single most common finding (about 4% of cases), and candidate genes include those for three GABA-A receptor subunits, GABRα5, GABRβ3, and GABRγ3 (Schroer et al., 1998). Correlating with neuropathology in the hippocampus of autistic individuals, the $\alpha5\beta3\gamma2$ subunit combination of GABA-A receptors predominates on hippocampal pyramidal cells, making up 20 percent of the GABA-A receptor population on these cells (McKernan and Whiting, 1996; for a review of GABA-A subunit combinations throughout the brain, see Whiting et al., 2000). An additional subunit combination of GABA-A receptors localized to GABA interneurons ($\alpha1\beta2\gamma2$) (Whiting et al., 2000) has a widespread distribution in the brain (approximately 50% of the total GABA-A receptor population). It is unclear whether any of these candidate genes are affected in cases with this chromosomal abnormality or in other cases of autism, but it is interesting to speculate, given the more widespread involvement of the GABAergic system, that subunit gene(s) for GABA-A receptors are likely target(s) in autism (e.g., subunit switching at specific times during development, possibly due to neuronal loss in specific subcortical or cortical nuclei). Interestingly, Shao et al. (2003) applied an innovative new statistical genetic approach to families whose children scored high in the "insistence on sameness" character trait (extreme diffi-

culty with changes to their daily routine) and who exhibit "repetitive compulsions." The researchers discovered a strong link between these phenotypic behaviors and the GABRβ3 locus in the chromosome 15q11–q13 region in patients with autism.

Future Directions

More experimental evidence is needed in neuropathologically affected and unaffected brain regions to better define and understand the effects on the GABAergic system in the autistic brain. Many key questions involving key afferent-target interactions may provide developmental and connectional clues to what neuroanatomic and functional anomalies are present in autistic individuals. In the cerebellum, a fascinating question is where olivocerebellar climbing fibers distribute due to the absence of many of their key targets, the Purkinje cells. What is the state of the remaining Purkinje cells: Are they functionally normal? Are they hyperinnervated by basket cell plexuses and/or by climbing fibers? Do climbing fibers function mainly through the cerebellar nuclei? Recent markers now commercially available may provide answers to some of these mysteries. Antibodies for climbing fibers, such as peripherin, can be used to follow these afferents through the white matter and granule cell layers into the Purkinje and molecular layers in the autistic brain. Perpherin labels the class III intermediate filament protein, peripherin, which is specific for climbing fibers but also labels the spinocerebellar and vestibulocerebellar mossy fibers terminating in the granule cell layer (Errante et al., 1998). Additionally, a functional marker for high-affinity GABA uptake sites (e.g., GAT-1) can be used to label the GABAergic basket cell plexus in combination with the classic silver stain that structurally labels the same perisomatic nest surrounding Purkinje cell soma. These markers will address whether "empty baskets" persist in the absence of many Purkinje cells. The studies of Fatemi et al. (2002) demonstrating a 50–51 percent decrease in the GABA synthesizing enzymes, GAD65 and GAD67, need to be followed up with in situ hybridization studies to localize these changes to specific cell types in particular cerebellar lobules.

In the limbic system, our initial findings (Blatt et al., 2001) need to be extended to other receptor ligands, such as toward the GABA-B receptor and to specific GABA-A subunit combinations, to determine whether there is a wider extent of involvement of the GABAergic system in the hippocampus, cerebellum, and beyond (e.g., the amygdala). Recent studies continue to reveal the distribution of GABA-A subunit combinations in key brain regions, including the limbic system and cerebellum (for a review, see Whiting et al., 2000), and thus, molecular studies can be directed toward specific receptor subunits in autism to

determine whether any alteration in the density and distribution occurs in affected areas. Additionally, populations of GABAergic interneurons are now being quantitatively characterized in the hippocampus with antibodies specific to subpopulations, which may reveal particular pathways, and/or receptor types that may be altered in autism. The hope is that studying the GABAergic system in autism will lead to early intervention therapy in an attempt to circumvent some of the early developmental events in the autistic brain.

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24

The Cholinergic System in Autism

Elaine Perry, Ph.D., and Mandy Lee, B.S.

There is as yet no etiology-based treatment or cure for autism. Neurotransmitter signaling systems are relevant to symptom etiology, treatment, and brain development. Transmitters so far implicated in autism include the monoamines—serotonin, dopamine, noradrenaline—together with acetylcholine, gamma-aminobutyric acid (GABA), glutamate, and several neuropeptides. Investigations have mainly relied on measurements in blood or cerebrospinal fluid or responses to pharmaceutical agents, and more recently, on genetic linkage data and observations using human brain tissue.

The cholinergic system is the most complex of the modulatory transmitter systems in the human brain (Figure 24.1), consisting of neurons in the basal forebrain projecting to the hippocampus, cortex and thalamus, and also striatal interneurons and pontine neurons projecting to the reticular formation and thalamus. The cholinergic system has been implicated in autism on the basis of pathologic abnormalities reported in the basal forebrain (septal) cholinergic neurons, such as increased and decreased size and numbers in, respectively, younger and adult individuals (Bauman and Kemper, 1994).

Cholinergic afferents innervate the cerebral cortex during the most dynamic periods of neuronal differentiation and synapse formation, suggesting that they play a regulatory role in these events (Hohmann and Berger-Sweeney, 1998). In the rodent cortex, the cholinergic innervation is the last of the modulatory afferent innervations to mature (Uhlings et al., 1992). In the human cerebral cortex and hippocampus, cholinergic activities, including choline acetyltransferase (ChAT, the enzyme synthesizing the transmitter) and high-affinity nicotinic receptor binding, alter substantially in the early postnatal period, when symptoms of autism first manifest (Court et al., 1993, 1997; Lainhart and Piven, 1995). Disruption of cholinergic innervation during early postnatal development (e.g., neonatal basal forebrain cholinergic lesions in rats) results in delayed cortical neuronal development and permanent changes in cortical cytoarchitecture and

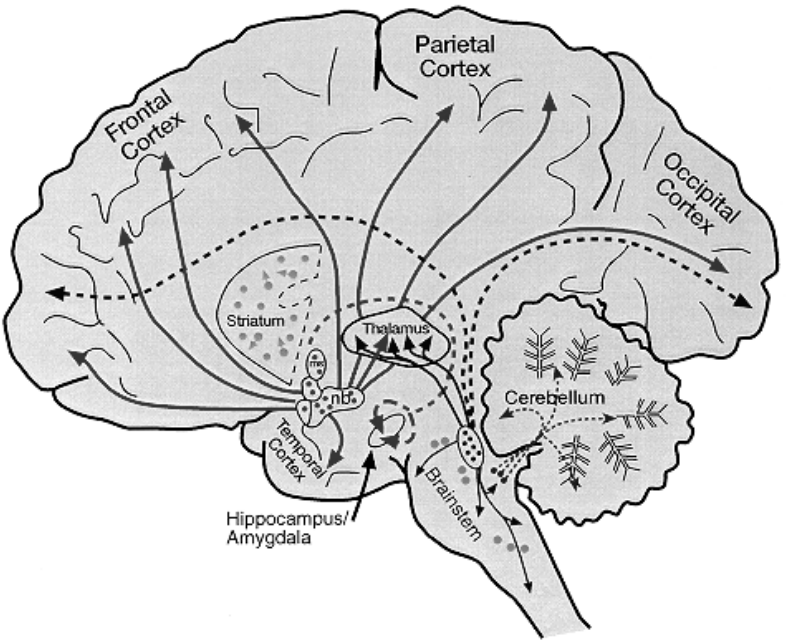

FIGURE 24.1. Cholinergic system in the human brain.

cognitive function (Hohmann and Berger-Sweeney, 1998). Abnormalities in cortical cytomorphology, including altered thalamic afferent distribution in layer IV (Hohmann et al., 1991a, 1991b; Sengstock et al., 1992), resemble pathologies associated with developmental disorders resulting in mental retardation (Huttenlocher, 1975).

This chapter focuses on recent neurochemical investigations of the cholinergic system in autism with potential implications for etiology, symptomatology, and therapy.

Cholinergic Neurochemical Activities in the Cerebral Cortex

PRESYNAPTIC ACTIVITIES

No abnormalities are apparent in the activity of the enzyme ChAT (which synthesizes acetylcholine) in frontal or parietal cortex or the basal forebrain, although activity in the basal forebrain tends to be increased (Perry et al., 2001). In relation to the original neuropathologic evidence of basal forebrain cholinergic dysfunction in autism (reduced neurons in adult autistics) (Bauman and

Kemper, 1994), the finding of normal ChAT both in frontal and parietal cortex and in the basal forebrain suggests that presynaptic cholinergic innervation of the cortex is structurally intact in autism. This indicates either that cholinergic neurons are not depleted in the cases examined or that compensatory axonal sprouting has occurred in conjunction with cell loss. In another developmental disorder, Rett syndrome, in which ChAT and the vesicular acetylcholine transporter (also a presynaptic marker) are reduced in various areas including the cortex (Wenk et al., 1991; Wenk and Mobley, 1996), disruption of cholinergic innervation is likely due to developmental or degenerative neuronal abnormalities occurring before or shortly after birth in the absence of compensation. The contrast with autism is striking, and elevated brain-derived neurotrophic factor (BDNF) in autism (see below) may perhaps play a role in maintaining cortical cholinergic projections.

NEUROTROPHINS

Of the two neurotrophins that control cholinergic function investigated in the basal forebrain, nerve growth factor (NGF) levels are not significantly changed, but BDNF levels are significantly elevated (threefold) (Perry et al., 2001). This increase is also apparent in the cerebral cortex, where although overall BDNF levels are lower than in the basal forebrain, they were more than fivefold higher in six autistic individuals compared to five control cases (Perry et al., unpublished data). The finding of increased BDNF in the basal forebrain and cortex can be interpreted variously. BDNF plays a role in sculpting synaptic connections. Because BDNF is up-regulated by cholinergic activity in developing rat hippocampus (da Penha Berzaghi et al., 1993), it is possible the abnormality is due to a transient developmental cholinergic hypertrophy. Another possible explanation for the elevation, because neurotrophins influence the development and function of basal forebrain cholinergic neurons (Hohmann and Berger-Sweeney, 1998; Hashimoto et al., 1999), is that it reflects a regional compensatory mechanism. This may underlie the finding that cholinergic biomarkers, such as ChAT in the cortex and basal forebrain, are normal or even elevated. A further explanation is that overexpression of BDNF is an intrinsic component of the autism disease process. Recent findings of elevated blood levels of BDNF (among three other brain peptides or proteins, including vasoactive intestinal peptide and calcitonin gene-related peptide) in newborn autistic and other mentally retarded individuals (Nelson et al., 2001) suggest an intrinsic rather than compensatory mechanism. BDNF could also be relevant to epilepsy, because decreased BDNF signaling in transgenic mice reduces epileptogenesis (Lahteinen et al., 2002).

MUSCARINIC RECEPTORS

Binding to the M2 muscarinic receptor (which is located presynaptically on a variety of neuronal types) is normal in frontal and parietal cortex in autism (Perry et al., 2001). Cortical muscarinic M1 receptor binding in the same areas is, in contrast, according to one report, decreased (Perry et al., 2001). M1 receptor loss was apparent in frontal and parietal cortices and in both outer and inner cortical layers, and reached significance in the parietal cortex. This neurochemical abnormality may be specific to autism, because it is not apparent in nonautistic mentally retarded individuals. The receptor abnormality reflects an alteration in the number and not affinity of the binding site, because IC_{50} values for pirenzepine displacement are similar in autistic compared to control cases (Perry et al., 2001).

Reduced M1 muscarinic receptor binding in the parietal cortex in autism indicates a specific abnormality in cholinoceptive function, because the M1 receptor is located postsynaptically. This could be related to epilepsy, which occurs in up to 40 percent of autistic children (Minshew et al., 1997), because a loss has been reported in hippocampal sclerosis associated with temporal lobe epilepsy (Pennell et al., 1999). The finding of normal pirenzepine binding in a similar series of cases in the hippocampus (Blatt et al., 2001), an area particularly susceptible in epilepsy, suggests another basis for the receptor loss, such as dendritic dysfunction.

NICOTINIC RECEPTORS

Nicotinic receptors are ligand gated channels (Na^+ and Ca^{2+}) consisting of a variety of α and β subunits. The principal subtypes in human brain are the α4β2 and α7 homomers (Figure 24.2). With respect to nicotinic receptor binding, there is no alteration of α-bungarotoxin (αBT) binding in the cerebral cortex (Perry et al., 2001). By contrast, in almost all cortical areas so far examined (frontal, parietal, and occipital, but not cingulate) significant and extensive reductions of epibatidine binding (to 20%–30% of the normal) are apparent throughout the different cortical layers (Figure 24.3). Epibatidine binding is also significantly reduced in nonautistic mentally retarded individuals but not in Down syndrome. It is unlikely that the receptor loss is due to differential use of tobacco between the groups, because in the basal forebrain, [^{3}H]nicotine binding is normal.

Similar to the M1 receptor abnormality, the nicotinic receptor abnormality reflects a reduction in receptor number, not affinity. Based on Western blotting

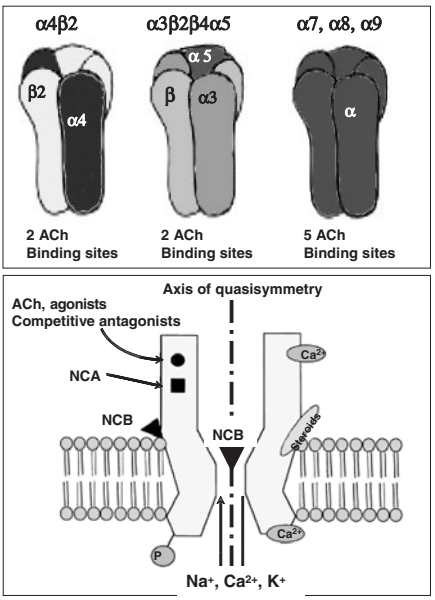

FIGURE 24.2. Neuronal nicotinic receptors. NCA, noncompetitive allosteric site; NCB, noncompetitive channel blocker; ACh, acetylcholine; M1, M2, M3, M4, muscarinic receptor subtypes. *Source:* Figure derived from Paterson and Nordberg (2000).

in the cortex, a selective loss of α4 and β2, but not α3 or α7 immunoreactivities is apparent in autism (Martin-Ruiz et al., 2000). Alpha 4 messenger RNA (mRNA) detected using RT-PCR in conjunction with GAPDH-1 housekeeper mRNA (Martin-Ruiz et al., 2004) is also reduced to the same extent as the receptor binding. This suggests that the abnormality exists at the level of gene expression and raises the question of whether there are any abnormalities of the α4 subunit gene (CHRNA 4) or its promoter in autism.

CHOLINE

Some recent in vivo imaging evidence suggests that choline levels may be increased in autism. Hydrogen proton magnetic resonance spectroscopy indicates an elevation in the choline:creatinine ratio related to disease severity (Sokol et al., 2002). This may reflect disturbed phospholipid metabolism related to membrane integrity or disturbances in cholinergic transmission.

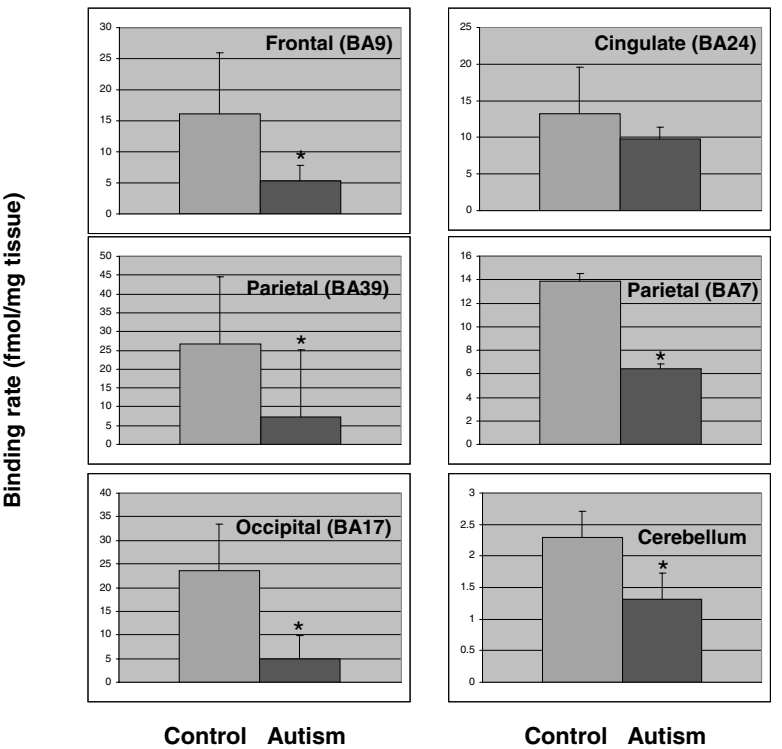

FIGURE 24.3. Nicotinic receptor (epibatidine) binding in autism. The asterisk indicates $p = 0.05$ (six autistic cases and eight controls).

Cholinergic Neurochemical Activities in the Cerebellum

CHOLINERGIC INNERVATION OF THE CEREBELLUM

The cerebellum is frequently implicated in autism on the basis of neuropathologic and imaging abnormalities. The cholinergic innervation of the cerebellum is complex. The predominant cholinergic input originates from the vestibular nuclei (Barmack et al., 1992). Other projections include (in the gerbil brain) those originating in the midline medullar periventricular gray, the C3 adrenergic area raphe obscurus nucleus, and in a variety of the reticular formation nuclei, together with various sensory nuclei (Lan et al., 1995). In the cat brain, cholinergic tegmental cerebellar projecting neurons occur in the pedunculopontine and lateral dorsal tegmental as well as locus coeruleus nuclei (Cirelli et al., 1998). In the rat, a subset of cerebellar mossy fibers, rich in ChAT, originates from the medial vestibular nucleus and innervates unipolar brush cells

(Jaarsma et al., 1996) and granule cells (Jaarsma et al., 1997). The granule cell layer of the rat vestibulocerebellum contains nicotinic binding sites (Jaarsma et al., 1996). Based on autoradiographic analysis in human cerebellum, nicotine binding has been detected in molecular and granule cell but not Purkinje cell layers (Court et al., 1995). In the rat cerebellum, nicotinic acetylcholine receptor (nAChR) α4 subunit immunoreactivity has been identified in cell bodies in the molecular, granule, and Purkinje cell layers and in axon terminals forming synapses with Purkinje cells (Nakayama et al., 1997). Alpha 7 nAChR subunit immunoreactivity is apparent in rat Purkinje cells and granule cell dendrites in glomeruli, but not in granule cell somata (Caruncho et al., 1997).

DEVELOPMENTAL CHANGES IN CEREBELLAR CHOLINERGIC ACTIVITIES

Paralleling developmental changes in ChAT, cerebellar cholinergic receptors decline postnatally. Muscarinic M2 and nicotinic receptors are higher in fetal than in adult human cerebellum (Court et al., 1995). Messenger RNA for α4, α5, α7, β2, and β4 but not α3 nAChR subunits are also higher in prenatal than in adult human cerebellum (Hellström-Lindahl et al., 1998; Hellström-Lindahl and Court, 2000). In the rat brain, M1, M3, and M4 muscarinic receptor subtypes decrease from juvenile to adult (Tice et al., 1996).

PRESYNAPTIC CHOLINERGIC ACTIVITIES

In autism, there is no alteration in cerebellar ChAT or M2 receptor binding, as is true of the cerebral cortex (Lee et al., 2002). By contrast, in Rett syndrome, ChAT is reduced in the cerebellum, as in other areas (Wenk et al., 2004). In another disorder involving cerebellar dysfunction, olivopontocerebellar atrophy, presynaptic cholinergic activities are also affected, to judge from reductions in acetylcholinesterase, which is partially presynaptic (Kish et al., 1989). As in the cerebral cortex, presynaptic cholinergic structures appear to be intact in autism, whereas nicotinic receptor changes (see below) are likely to reflect abnormalities in cholinoceptive neurons or in noncholinergic presynaptic structures.

RECEPTOR BINDING

Muscarinic M1 and M2 receptors are not affected in the cerebellum in autism, with the exception of an elevation in M1 levels, which are, however, normally extremely low in this brain area.

There is a significant reduction of up to 50 percent in epibatidine binding in autism compared to normal control group in all layers of the cerebellum (Lee et al., 2002). In contrast, αBT binding is elevated in the autism compared to control group, with a significant threefold increase in the granule cell layer. This α7 nicotinic receptor increase appears to be specific to autism, not being apparent to the same extent in a nonautistic mental retardation group. The αBT binding predominantly reflects the α7 subunit, and this abnormality is of interest, because the gene encoding this subunit is located close to q11–15 on chromosome 15 (Chini et al., 1994), near the portion of this chromosome associated with abnormalities in autism (Lamb et al., 2000). However, mRNA levels for α4 and α7 subunits in the cerebellum were not significantly altered in autism, despite a tendency for both to be elevated (Martin-Ruiz et al., 2000). This suggests a different etiopathology for the nicotinic receptor abnormalities in the cerebellar and cerebral cortices.

NICOTINIC RECEPTOR IMMUNOCHEMISTRY

Western blotting in the cerebellum indicates that the α4 subunit is significantly reduced, and α7 is increased, although not significantly (Martin-Ruiz et al., 2000; Lee et al., 2002).

Immunohistochemically, α4 nAChR is apparent throughout the cerebellar cortex, with diffuse immunoreactivity in the neuropil of the molecular layer and granule cell layers. Stellate cells of the molecular layer, occasional large cell bodies (possibly Golgi cells in the granule cell layer), and a small proportion of granule cell soma are α4 immunoreactive. Alpha 4 immunoreactivity in Purkinje cells extends into branching apical dendrites. Strong coarse granular α4 immunoreactivity is present in the neuropil surrounding the Purkinje cells and also in and between neurones of the deep cerebellar (fastigial and dentate) nuclei. In autistic cases, α4 immunoreactivity is decreased in all layers of certain areas of the cerebellar cortex, particularly in the culmen and declive of the cerebellar vermis (Lee et al., 2002). In these lobes, α4 immunoreactivity is lost from the neuropil of the molecular and granule cell layers. Stellate and granule cell soma appeared to be decreased and granular immunoreactivity markedly reduced in the granule cell and Purkinje cell layers. Alpha 4 immunoreactivity in the deep cerebellar nuclei appears to be unaffected.

Diffuse α7 nAChR subunit immunoreactivity extends throughout the layers of the normal cerebellar cortex and the deep white matter. Purkinje cells have variable α7 immunoreactivity, sometimes extending into apical dendrites. Granular immunoreactivity occurs in the neuropil surrounding the Purkinje cells and between α7 immunoreactive neurons in the deep cerebellar nuclei. In

the autistic cases examined, there is a reduction in diffuse α7 immunoreactivity throughout the layers of the cerebellum cortex. Purkinje cells and stellate cells also have decreased immunoreactivity. In contrast, granule cells in certain areas of the autistic cases showed an increase in α7 immunoreactivity. The apparent increase in α7 immunoreactivity in the granule layer may reflect compensatory up-regulation during development.

Implications of Nicotinic Receptor Loss in Relation to Neuronal Plasticity

Because nicotinic receptors are thought to play a particular role in regulating synaptic/dendritic plasticity, it is likely that the receptor reduction in autism relates to this aspect of neuronal function. In the development of retinal ganglion cells, for example, exposure from early embryonic stages to the nicotinic antagonist, curare, aborts dendritic proliferation (Sernagor et al., 2001).

Relationships between loss of the α4 nicotinic receptor subtype and synaptophysin identified in the cerebral cortex in another cerebral disorder, Alzheimer disease (Sabbagh et al., 1998), suggest that the receptor loss in autism may be associated with abnormal synaptic morphology and function, possibly involving overextensive synaptic pruning during development.

Knockout (β2) mouse models, lacking high-affinity agonist receptor binding, develop a degree of age-related cortical atrophy and neuronal loss in conjunction with cognitive impairment (Picciotto et al., 1999; Table 24.1). Loss of the high-affinity nicotinic receptor agonist site not only in autism but also in the non-autistic mental retardation group could indicate a lack of specificity to autism; it could also indicate that the receptor loss may be a consequence rather than a cause of cortical dysfunction. However, developmental brain abnormalities occur in both groups, and an overlap in the processes involved would be expected. It is important to determine at what age the receptor abnormality in autism occurs. This could be investigated using novel nicotinic receptor ligands applicable to positron emission tomography or single proton emission computed tomography in vivo imaging.

In interpreting the nicotinic receptor abnormalities in the cerebellum, it is relevant that neurogenesis in the cerebellum occurs at approximately the fifth week of gestation, which period may represent a “window” of vulnerability for autism (Courchesne, 1997). Prenatal exposure of rats to valproic acid results in significantly fewer Purkinje cells in the embryonic cerebellar vermis (Ingram et al., 2000). From other studies on the developing rat cerebellum, it is apparent that an increase in Purkinje cell α7 immunoreactivity coincides with major

TABLE 24.1. nAChR Subunit Knockouts and Knockins Relevant to CNS Function

Subunit	*Effect*	*Reference*
α_4 knockout	Lack of nicotine antinoceptive response	Marubio et al. (1999)
	Increased basal level of anxiety	Ross et al. (2000)
	Loss of nicotine neuroprotection-metamphetamine treatment	Ryan et al. (2001)
α_4 knockin	Reduced dopamine function in aging in channel pore point mutation with hypersensitive receptors+	Labarca et al. (2001)
β_2 knockout	Attenuation of nicotine self-administration	Picciotto et al. (1995)
	Lack of antinoceptive response	Marubio et al. (1999)
	Impaired spatial learning in aging	Zoli et al. (1999)
	Cortical atrophy and neuron loss in aging	Zoli et al. (1999)

developmental synaptogenic events (Dominguez del Toro et al., 1997). It has also been suggested that the α7 subunit mediates developmental plasticity in the chick cerebellum (Kaneko et al., 1998).

Nicotinic receptors in both the cerebral cortex and cerebellum govern the release of other transmitters, such as GABA or glutamate. The presence of both α4 and α7 subunits on Purkinje cells and their dendrites in normal adult cerebellum, for example, is consistent with localization of nAChRs on this GABAergic neuronal population. Punctate immunoreactivity in both Purkinje and granule cell layers suggests that a proportion of the receptors in the cerebellar cortex is located presynaptically, modulating the release of, for example, glutamate from mossy fibers (Didier et al., 1995). The alterations in α4 and α7 subunits observed in autism are thus likely to result in alterations in such types of noncholinergic neurotransmission.

Comparisons with Cholinergic Pathology Occurring in Other Disorders

The overall pattern of cholinergic abnormalities in autism—loss of nicotinic and, to a lesser extent, muscarinic receptors in conjunction with normal presynaptic cholinergic markers in the cortex—is more similar to that seen in schizophrenia than in other disorders involving a cholinergic abnormality, such as Rett

syndrome, or age-related disorders, such as Alzheimer or Parkinson disease, head injury, or vascular dementia (Table 24.2). There is, in common with schizophrenia, a sparing of the presynaptic marker, ChAT, but reductions in muscarinic and nicotinic receptors, although in schizophrenia, the α7 subtype may be more affected than the α4 (Perry and Perry, 1980; Freedman et al., 1995). In schizophrenia, as in autism, there is also an elevation in BDNF but not NGF in cortical areas (Takahashi et al., 2000). There is an overlap in clinical symptoms between autism and schizophrenia, both behaviorally and cognitively (e.g., in conceptual abnormalities) (Pilowsky et al., 2000). The same neural systems are likely to be involved in both, although differing in developmental staging and etiology.

Clinical Implications of the Nicotinic Receptor Loss

Although not yet established, nicotinic receptor loss could relate to clinical features of autism, such as attentional abnormalities, pain perception, anxiety, or perhaps epilepsy. So far, it does not appear that the receptor loss is associated with epilepsy (Perry et al., 2001). The cholinergic system has long been implicated in attention (for reviews, see Perry et al., 1999; Sarter and Bruno, 2000), with a specific role for the nicotinic receptor (Mirza and Stolerman, 2000). Nicotine administered accurately in humans results in improvements in

TABLE 24.2. Cortical Cholinergic Activities in Cerebral Disorders

Disorder	*ChAT*	*M1*	*M2*	*nAChR*		*BDNF*
Autism	→	↓	→	↓		↓
Down						
Young		→	→	→	↓	
Old		↓			↓	
Rett	↓					
Schizophrenia	→	↓		↓?		↓
Alzheimer	↓	→	↓	↓		↓?
Parkinson	↓	↑		↓		
DLB	↓	↓		↓		
Head injury	↓	→	→	→		
Cerebrovascular	↓			→?		

Notes: BDNF, brain derived neurotrophic factor; ChAT, choline acetyltransferase; nAChR, nicotinic acetylcholine receptor; DLB, diffuse Lewy body disease.

extended vigilance tasks, divided attention, and rapid information processing (K. Wesnes, personal communication). The receptor loss may thus relate to attentional deficits in autism. Nicotinic agents are analgesic (Bannon et al., 1998), and on the basis of a gene knockout model (Marubio et al., 1999: Table 1) the α4 subunit has been implicated in pain perception. Low levels of this receptor subtype in autism may thus be associated with reduced pain reactivity, which occurs in the disease (Tordjman et al., 1999).

Abnormal galvanic skin responses (threefold greater peak amplitudes, double the normal number of arousal events per minute, and the absence of a baseline) have been observed in autism (P. Iversen and H. Ramshandran, unpublished data). Because these may depend on the integrity of sympathetic cholinergic neural pathways (Magnifico et al., 1998), it may be worth investigating the extent to which the central nervous system cholinergic abnormalities reported here extend to the peripheral nervous system.

Therapeutic Implications

There is no U.S. Food and Drug Administration–approved pharmacotherapy for autism, although neuroleptics, benzodiazepines, anticonvulsants, and selective serotonin reuptake inhibitors are prescribed symptomatically. There is currently one report that cholinesterase inhibitors (donepezil and rivastigmine) administered to autistic children result in symptomatic improvement, especially language, in 70 percent of cases (Chez et al., 2004). Particularly encouraging, symptoms did not revert on cessation of drug treatment, consistent with the concept that restoring deficient cholinergic neurotransmission is of not only short-term but also long-term benefit, related perhaps to neurotrophic effects of such chemical transmitters as acetylcholine (Belluardo et al., 2000). Controlled clinical trials of currently available and developing cholinergic therapies in autism are warranted.

If the nicotinic receptor loss in autism is consistently observed and clinically relevant, therapeutic strategies could include receptor agonists, such as nicotine, provided via patches or inhalers. Nicotine has already been administered in Tourette syndrome, with amelioration of symptoms (Sandberg et al., 1997). Such treatment could also be disease modifying. Nicotine administration in animal models or tobacco use in humans results in an increase in nicotinic receptor levels (particularly α4) (Martin-Ruiz et al., 1999). Nicotine significantly increases branching of both axons and dendrites in cultured hippocampal neurons (Audersirk and Cabell et al., 1999). In the adolescent rat brain, nicotine exposure results in persistent up-regulation of nicotinic receptors in a variety of brain areas, including the cortex (Trauth et al., 1999), and in the adult mouse brain, chronic nicotine exposure promotes retention of nicotinic receptors that decline

with age (Rogers et al., 1998). New nicotinic drugs targeted to the α4β2 receptor subtype exist and should be worth testing in clinical trials, assuming they are not associated with addictive behavior.

Neurochemistry of Consciousness

Autism has been described as "a disorder of consciousness and feelings" (Trevarthen et al., 2000), and restricted and repetitive behaviors have been interpreted as indicative of nonreflective thinking or absence of higher consciousness. Although there is no unanimity on the core cognitive characteristic in autism, it is interesting that the principal abnormality highlighted in this chapter—a lower level of the α4 nicotinic receptor—has already been linked to abnormalities in consciousness (Perry et al., 2002). Thus, neuronal nicotinic α4β2 receptors are inhibited at clinically relevant concentrations by a range of general anesthetic agents (Flood and Role, 1998; Tassonyi et al., 2002). Disturbances of consciousness are common in a disorder of the ageing brain, dementia with Lewy bodies. Such disturbances include fluctuating attention and awareness. In patients prospectively assessed clinically, this symptom is associated with differences in the level of the nicotinic α4-containing receptor subtype, but not in any other cholinergic, dopaminergic, or 5-hydroxytryptamine activity assessed (Ballard et al., 2002).

Table 24.3 summarizes other lines of evidence that support the concept of a role for acetylcholine in abnormal consciousness in autism. Thus, in addition to providing potential insights into etiopathology, neurochemical findings,

TABLE 24.3. Consciousness, Acetylcholine, and Autism

Observation	*Reference*
40-Hz neuronal synchronization essential neural correlate of consciousness (binding)	Engel and Singer (2001)
Acetylcholine, implicated in consciousness (Perry et al., 1999), induces 40-Hz synchronized oscillations in the hippocampus in vitro	Fisahn et al. (1998)
Disordered gamma (40 Hz) EEG in autism may relate to difficulties in integrating (binding) perceptual features	Grice et al. (2001)
Auditory evoked response abnormalities in autism may reflect dysfunction of cholinergic reticular activating system	Buchwald et al. (1992)

such as outlined here, may contribute new perspectives on the neurochemistry of consciousness.

Summary

The cholinergic system plays a key role in aspects of attention that are affected in autism. Evidence of cholinergic dysfunction in autism includes neuropathologic abnormalities originally reported in the basal forebrain cholinergic nuclei. In relation to neurochemical activities, although in the cerebral cortex and basal forebrain, there are no differences in ChAT or acetylcholinesterase activity, there are significant and extensive reductions (60%–75%) in the nicotinic receptor, assessed using [^{3}H]epibatidine binding in several areas of the cerebral cortex and cerebellum. Immunochemical and RT-PCR analyses indicate a selective loss of the α4β2 nicotinic receptor subtype. There is, on the contrary, an increase (threefold) in the α7 nicotinic receptor, binding αBT in the cerebellum. There are modest reductions (20%–30%) in muscarinic M1 receptor binding, confined to the parietal cortex. In the basal forebrain and cerebral cortex, there is an extensive (more than threefold) increase in the level of the neurotrophin BDNF, which controls neuronal activities, including cholinergic. These findings indicate the potential importance of specific cholinergic receptors and neurotrophins in developmental neurobiology and may provide new insights into the etiopathology of autism. Therapeutic intervention based on cholinergic receptor modulation is currently being explored, and results so far indicate positive and nonreversible improvements in function, especially language, using cholinesterase inhibitors.

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25

The Role of Reelin in Autism

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Autism is a debilitating neurodevelopmental disorder with childhood onset and a rising prevalence rate of 16–20 per 10,000 (Korvatska et al., 2002). There is a significant body of evidence pointing to genetic (Folstein and Rosen-Sheidley, 2002) and environmental (Rodier, 2000) factors in the causation of autism. A putative link between gene and environment and neuropathologic findings and abnormal behavior is an array of critical proteins that are responsible for orchestrating normal brain development. Reelin glycoprotein is such a protein, with important functions both during the embryonic stages of brain development and in adult life (Guidotti et al., 2000; Fatemi, 2001). An increasing number of recent reports implicate Reelin in the etiology of neurodevelopmental disorders like schizophrenia and autism (Fatemi, 2001, 2002; Costa et al., 2002).

Reelin is a secretory extracellular matrix protein (D'Arcangelo et al., 1997) that was discovered in 1995 (D'Arcangelo et al., 1995; Ogawa et al., 1995). Reelin is composed of 3,461 amino acids (deBergeyck et al., 1998), with a chemical structure containing a signal peptide followed by a N-terminal sequence similar to F-spondin and a hinge region upstream from eight Reelin repeats of 350–390 amino acids (deBergeyck et al., 1998). The Reelin protein ends with a highly basic C-terminal region, composed of 33 amino acids, which is essential for secretion (D'Arcangelo et al., 1997; deBergeyck et al., 1998). Another important epitope localized near the N-terminus of Reelin is the CR-50, recognized by CR-50 antibody (Ogawa et al., 1995). The CR-50 epitope is composed of amino acids 230–346 of Reelin protein (Utsunomiya-Tate et al., 2000) and is essential for Reelin-Reelin electrostatic interaction, which causes the homopolymerization of up to 40 or more monomers of Reelin (Utsunomiya-Tate et al., 2000). A mutated form of Reelin, which lacks a CR-50 epitope, is incapable of producing this homopolymer and unable to transduce the Reelin signal (Utsunomiya-Tate et al., 2000). The relative molecular mass of Reelin is 388 kDa; however, on sodium-dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), after reduction, it

appears as a band of approximately 400–410 kDa (deBergeyck et al., 1998; Fatemi et al., 2001). Additionally, several putative cleavage products of Reelin seen on SDS-PAGE following reduction are bands of 330 kDa, 180 kDa, and smaller molecular weights (Smalheiser et al., 2000; Fatemi et al., 2001; Perez-Costas et al., 2002). A recent report indicates that Reelin possesses serine protease activity (Quattrochi et al., 2002), being able to enzymatically cleave laminin and fibronectin in vitro and cause modification of basal lamina structure (Quattrochi et al., 2002). The Reelin molecule itself may be protealytically processed by an unknown zinc-dependent enzyme (deRouvroit et al., 1999), resulting in several fragments whose biological functions remain unknown.

The Reeler mutant mouse, which carried an autosomal recessive mutation in the Reelin (*RELN*) gene, was discovered by Falconer (1951). The mutant mouse exhibited ataxia and a reeling gait. Examination of the brain in these mice showed multiple defects, including inverted cortical lamination, abnormal positioning of neurons, and aberrant orientation of cell bodies and nerve fibers (Falconer, 1951; Goffinet, 1979). In the cerebral cortex of the Reeler mouse, late-developing neurons that are usually destined to form the cortical plate fail to bypass previously generated neurons and remain in deep locations beneath their predecessors (Goffinet, 1979). Thus, an inverted pattern of cortical development takes place in the mutant animal. Moreover, the preplate fails to split in the Reeler mouse, resulting in malpositioning of subplate neurons close to the pial surface in a so-called "superplate layer." Additionally, profound cerebellar hypoplasia is evident in the brains of Reeler mice, with Purkinje cells remaining in deep clusters, failing to form the Purkinje cell plate, resulting in a dramatic reduction in the size of the cerebellum and a lack of foliation (Caviness and Sidman, 1973; Goffinet, 1984). Ectopic expression of Reelin in the Reeler mouse rescues cerebellar development and ataxia in the mutant mouse, indicating that Reelin protein is also important in correct cell positioning (Magdaleno et al., 2002).

Reelin cDNAs have been cloned from mouse (Bar et al., 1995; D'Arcangelo et al., 1995) and man (DeSilva et al., 1997). The gene for Reelin protein is localized on chromosome 7 in humans (DeSilva et al., 1997). Reelin RNA is first detectable in the mouse embryonic brain on day 9.5 (Ikeda and Terashima, 1997). The amount of Reelin RNA increases progressively up to early postnatal days and then declines to adult levels. The first cells that synthesize Reelin are the Cajal-Retzius cells, which begin differentiation as early as day 9.5 in the embryonic mouse brain (Ogawa et al., 1995). Cajal-Retzius cells are neurons that act as pathfinders and help in the early laminar organization of the cortex (Ogawa et al., 1995). In the adult mammalian brain, Reelin is localized to layer I Cajal-Retzius cells, cortical gamma-aminobutyric acid (GABA)-ergic inter-

neurons in layers II through VI (Impagnatiello et al., 1998; Pesold et al., 1998), cerebellar granular cells (Lacor et al., 2000), and hippocampal interneurons (Fatemi et al., 2000a). Reelin is also present in a number of other sites, such as spinal cord (Yip et al., 2000; Carrol et al., 2001), subpial granular layer of human fetal cortex (Meyer and Wahle, 1999; Phelps et al., 2002), developing rat striatum (Nishikawa et al., 1999), hepatic stellate cells (Kobold et al., 2002), human esophageal carcinoma (Wang et al., 2002), larval sea lamprey brain (Perez-Costas et al., 2002), human and mouse odontoblasts (Buchaille et al., 2000; Heymann et al., 2001), adult vertebrate pallium (Perez-Garcia et al., 2001), and blood, liver, and adrenal chromaffin cells and pituitary pars intermedia (Smallheiser et al., 2000; Fatemi et al., 2001).

Following the original discovery of Reelin protein (D'Archangelo et al., 1995; Ogawa et al., 1995), a number of other mutations have been described that exhibit Reeler-like phenotypes (Fatemi, 2001). These mutations include the mouse disabled-1 gene mutation (Gallagher et al., 1998), Scrambler mouse mutation (Sheldon et al., 1997), Yotari mouse mutant (Kojima et al., 2000), EMX2 mutant mouse (Mallamaci et al., 2000), VLDLR and ApoER2 double knockout mouse (Trommsdorff et al., 1999), presenilin-1 deficient mouse (Hartmann et al., 1999), rat mutation creeping (Yokoi et al., 2000), and the T-box transcription factor knockout mouse (Hevner et al., 1999). Additionally, a number of other animal models using various other mutations or experimental insults cause the production of defects in Reelin synthesis concomitant with cortical and behavioral abnormalities (Fatemi, 2001). Some of these include the heterozygous Reeler mouse (Tueting et al., 1999); following prenatal human influenza viral infection (Fatemi et al., 1999, 2002a); after x-irradiation (Darmanto et al., 2000) or domoic acid lesions of Cajal-Retzius cells (Super et al., 2000); in experimental hypothyroidism (Alvarez-Dolado, 1999); or after thromboxane A2 treatment (Fukami et al., 2000). Of great interest are the anatomic, biochemical, and behavioral deficits that have been observed in two of the abovementioned models: in heterozygous Reeler mutation (Costa et al., 2002) and in the progeny of mouse mothers exposed to human influenza viral infection on day 9 of pregnancy (Fatemi et al., 2002a; Shi et al., 2003), which resemble deficits seen in patients with schizophrenia and autism. For example, the heterozygate Reeler mouse exhibits increased cortical neuronal packing density, decreased neuropil density, altered distribution of NADPH (nicotinamide adenine dinucleotide phosphate)-diaphorase positive cells, decreased GAD67 (glutamic acid decarboxlase 67), messenger RNA (mRNA), and protein levels, decreased Reelin protein and mRNA levels, and abnormal prepulse inhibition (Tueting et al., 1999; Costa et al., 2002); abnormalities that exist in schizophrenia (Akbarian et al., 1993; Selemon and Goldman-Rakic, 1999; Guidotti et al., 2000) and, to some degree, in autism

(Fatemi et al., 2001, 2002b; Casanova et al., 2002). In the mouse progeny of mothers exposed to human influenza viral infection, the following abnormalities are seen: early thinning of the cortex and hippocampus, as well as reduced Reelin protein (Fatemi et al., 1999); altered distribution of neuronal nitric-oxide synthase protein in the exposed brains during various stages of brain development (Fatemi et al., 1998, 2000b); pyramidal cell atrophy and macrocephaly in the growing exposed mice (Fatemi et al., 2002a); and abnormal prepulse inhibition (Shi et al., 2003). Several of these abnormalities are also present in autistic subjects: macrocephaly (Miles et al., 2000), abnormal prepulse inhibition (McAlonon, Kumari, Geyer, Murphy, personal communication), pyramidal cell atrophy, and increased cell packing density (Casanova et al., 2002).

The Reelin protein binds several proteins to transduce its signals downstream. These receptors include apolipoprotein E2 receptor (ApoER2), very-low-density lipoprotein receptor (VLDLR), cadherin-related neuronal receptor (CNR) family and α3β1 integrin protein (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Senzaki et al., 1999; Trommsdorff et al., 1999; Dulabon et al., 2000; Stockinger et al., 2000). Reelin binding to its receptors facilitates the signaling pathway between Reelin-producing cells and their targets, on cortical pyramidal cells, causing activation of focal adhesion tyrosine kinase system (Grant et al., 1995). This kinase system is a component of a postsynaptic mechanism responsible for an increase in number of synapses and changes in postsynaptic spine structure in axons, dendrites, and the intermediate filament cytoskeleton of astrocytes. Additionally, Reelin also activates serine-threonine kinases (P35/Cdk5) and Src-tyrosine kinase, leading to phosphorylation of the adaptor protein Dab-1 (Keshvara et al., 2001). Phosphorylated Dab-1 may serve as a docking site for the SH2-domain of Src-kinase family of proteins whose activation may underlie synaptic and dendritic spine plasticity (Costa et al., 2001). It is also reported that the Reelin receptor ApoE2 protein is involved in recruitment of the C-JunN-Terminal kinases (JNK-interacting proteins) 1 and 2, subserving multiple functions, such as cell adhesion, vesicle trafficking, neurotransmission, and morphogenesis (Xia et al., 1995; Kuan et al., 1999).

Several reports implicate Reelin as being involved in different neuropsychiatric disorders (Impagnatiello et al., 1998; Fatemi et al., 2000a, 2001, 2002b; Guidotti et al., 2000; Hong et al., 2000; Persico et al., 2001). Costa and coworkers, through several landmark studies, showed for the first time that Reelin protein and mRNA were reduced in several brain sites in schizophrenic and psychotic bipolar patients (Guidotti et al., 2000; Costa et al., 2002). These authors suggested that Reelin might be a vulnerability factor in psychosis (Impagnatiello et al., 1998). Later, Fatemi et al. (2000a), while confirming Costa's findings in the hippocampus, detected similar reductions in nonpsychotic bipolar and

depressed patients, suggesting that Reelin deficiency may not be limited to subjects with psychosis. Further confirmatory data (Hong et al., 2000) showed that blood levels of Reelin were low to undetectable in two families with children suffering from a variant of lissencephaly. The affected children exhibited severe delays in neurologic and cognitive development (e.g., little or no language), hypoplastic cerebella, moderate lissencephaly, and generalized seizures (Hong et al., 2000). All affected children had various mutations involving the *RELN* gene. Overall, these reports clearly showed that Reelin deficiency was associated with a number of neurodevelopmental disorders whose central behavioral pathology involved cognitive dysfunction of variable severity (Fatemi, 2001).

Subsequently, Fatemi et al. (2001) determined the levels of Reelin protein in the cerebellar tissues of autistic subjects. Autistic and normal control cerebellar cortices matched for age, sex, and postmortem interval were prepared for SDS-gel electrophoresis and Western blotting using specific anti-Reelin antibodies. Quantification of Reelin bands showed 43 percent, 44 percent, and 44 percent reductions in autistic cerebellum (mean optical density ± standard deviation [SD] per 30-µg protein: 4.05 ± 4.0, 1.98 ± 2.0, 13.88 ± 11.9 for 410-kDa, 330-kDa, and 180-kDa bands, respectively; $N = 5$) compared to controls (mean optical density ± SD per 30-µg protein: 7.1 ± 1.6, 3.5 ± 1.0, 24.7 ± 5.0; $N = 8$; $p < 0.0402$ for 180-kDa band). Measurement of β-actin (mean ± SD for controls: 7.23 ± 2.7; for autistic subjects: 6.77 ± 0.66) in the same homogenates did not differ significantly between groups (Figure 25.1A,B). These results demonstrated for the first time that dysregulation of Reelin may be associated with some of the brain structural and behavioral abnormalities observed in autism (Rodier, 2000).

In a recent report, Fatemi et al. (2002b) hypothesized that blood levels of Reelin and its isoforms would be reduced in autistic-monozygotic twins and their first-degree relatives versus normal controls. They measured levels of unprocessed Reelin (410 kDa) and its proteolytic cleavage products (Reelins 330 and 180 kDa), as well as albumin and ceruloplasmin in 28 autistic individuals, their parents (13 fathers, 13 mothers), six normal siblings, and eight normal controls using SDS-PAGE and Western blotting (Fatemi et al., 2002b). Results indicated significant reductions in 410 kDa Reelin species in autistic twins (–70%; $p < 0.01$), their fathers (–62%; $p < 0.01$), their mothers (–72%; $p < 0.01$), and their phenotypically normal siblings (–70%; $p < 0.01$) versus controls. Reelin 330 kDa values did not vary significantly from controls. Reelin 180 kDa values for parents (fathers: –32%; mothers: –34%; $p < 0.05$ versus controls) declined compared to controls (Figure 25.1C). In contrast, autistic Reelin 180 kDa increased, albeit nonsignificantly versus controls. Albumin and ceruloplasmin values for autistic subjects and their first-degree relatives did not vary significantly from controls. There were no correlations between Reelin, albumin, and ceruloplasmin levels,

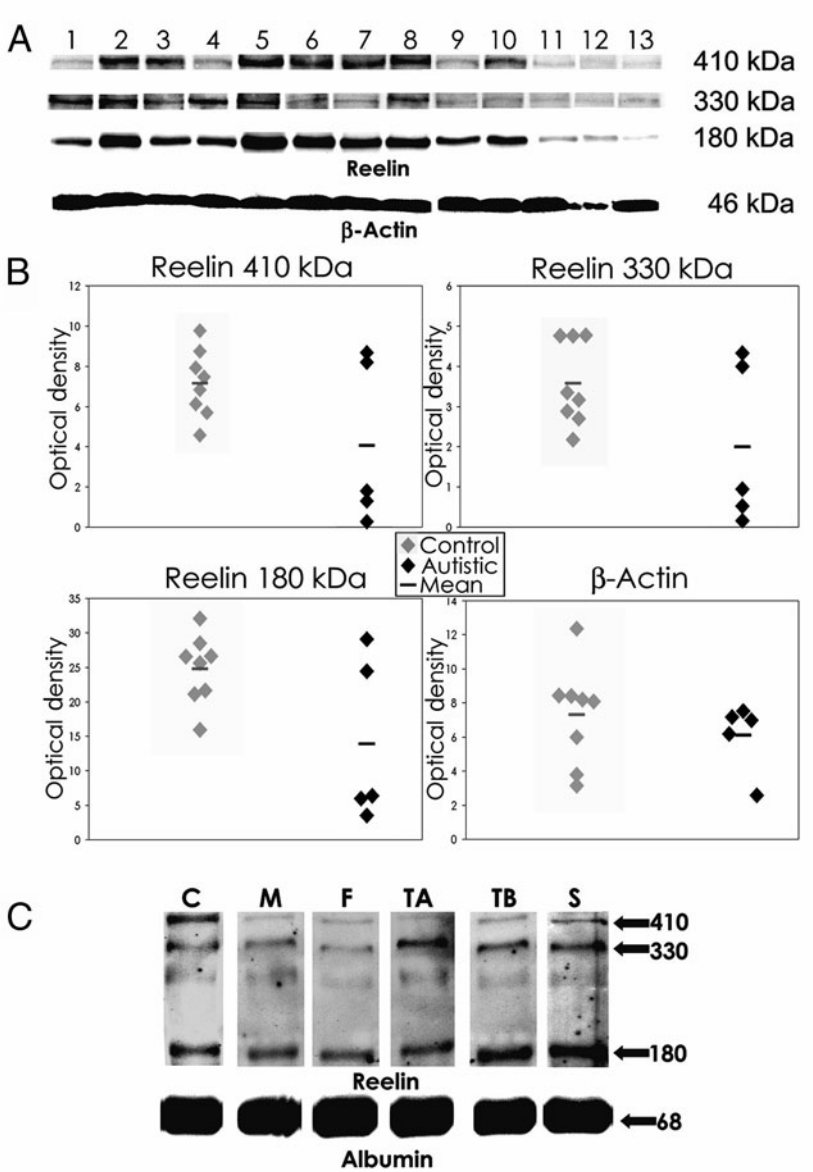

FIGURE 25.1. (A) Gel mobility and density of various Reelin bands 410, 330, and 180 kDa and β-actin in cerebellar homogenates (30-μg protein per lane) of control (lanes 1–8) and autistic (lanes 9–13) subjects. (B) Scatter plots for Reelin and β-actin levels in autistic subjects and controls, expressed as optical density values. Mean autistic Reelin 180 kDa is reduced significantly ($p < 0.04$) compared with controls. (C) Gel mobility and density of various Reelin bands, albumin, and ceruloplasmin (30 μg protein per lane, except for albumin 5 μg per lane) for representative normal control (C), mother (M), father (F), autistic twins (TA and TB), and normal sibling (S) are shown. Optical density units are arbitrary. *Source:* Fatemi et al. (2001).

age, sex, ADI (Autism Diagnostic Interview) scores or age of onset. These results demonstrated that Reelin 410 deficiency may be a vulnerability factor in the pathophysiology of autism (Fatemi et al., 2002b).

Confirmatory genetic data provided by Persico et al. (2001) also showed a significant association between autism and *RELN* gene variants using case-control and family-based designs. Persico et al. (2001) discovered a polymorphic GGC repeat located immediately 5′ to the ATG initiator codon of the human Reelin gene. Six distinct alleles, containing eight to 14 copies of the repeat, were found in the general population, and four additional alleles, containing four, seven, 15, and 23 repeats, were found in autistic patients and their first-degree relatives. Alleles containing eight to 10 repeats were most common in the general population, although genotypic and allelic distributions were different for different ethnic groups. Individuals with autism, however, tended to have more repeats. For Caucasians of Italian descent, 17.9 percent (17 of 95) autistic individuals versus 9.1 percent (17 of 186) unaffected persons had at least one allele containing 11 or more repeats, and the frequencies of "long" alleles (≥11 repeats) were also found in a study of 89 Caucasian Americans of European descent and their first-degree relatives (Persico et al., 2001). In addition to the polymorphic GGC repeat, Persico et al. (2001) described two additional polymorphisms that were informative for genetic analysis of the *RELN* locus: (1) a A/G transversion predicted to affect RNA splicing was detected within the acceptor site in intron 5 (immediately 5′ to exon 6); and (2) a conservative T/C transversion in the codon for histidine 2682 was detected in exon 50. Haplotype analysis using these three genetic markers revealed statistically significant increases in the frequency of occurrence in autistic patients versus controls for several specific combinations of alleles.

More recently, Zhang et al. (2002) tested the hypothesis that instability in the number of repeats in the *RELN* gene, or an altered distribution in these alleles, may be associated with increased susceptibility to the development of autism. These investigators examined the distribution of GGC repeat numbers in affected members from 126 multiplex and 68 simplex families and found no evidence for expansion or instability of transmissions of this repeat in the autistic subjects. The authors discovered 14 alleles of variable number of GGC repeats (three to 16 repeats) in the affected and control subjects (Zhang et al., 2002); there were no differences in the case-control studies for the most common alleles (i.e., eight or 10 alleles or genotypes).

Additionally, Zhang et al. (2002) did not observe an increased frequency of large alleles (≥11 repeats) in the autistic cases versus controls. Later, using a family-based association test, these authors showed that the larger alleles (≥12 repeats) were transmitted higher than expected in the affected children ($S = 43$;

$E(s) = 34.5$; $p = 0.035$) and in particular with the 13 repeat allele ($S = 22$; $E(s) = 16$; $p = 0.034$) (Zhang et al., 2002). This increased risk of developing autism supports the findings of Persico et al. (2001), without evidence of increased allele sharing previously described in the siblings of the autistic children. Although the case-control studies of Zhang et al. (2002) did not replicate Persico and colleagues' (2001) data, their family-based data support the latter's (2001) study, adding to the weight of genetic evidence implicating *RELN* in the etiology of autism (Krebs et al., 2002). Despite these positive findings, a recent study (Krebs et al., 2002) could not show any association between a polymorphic GGC repeat in the 5′ untranslated region of the *RELN* gene and autism in a population of mixed European descent, emphasizing the need for more rigorous genetic studies to establish a definitive link between the *RELN* gene and autism.

Reelin glycoprotein serves a dual purpose in the mammalian brain. Embryologically, it guides neurons and radial glial cells to their correct positions in the brain (Forster et al., 2002; Luque et al., 2003). After the fetal phase of development, levels of Reelin begin to decrease, reaching a plateau and remaining constant thereafter in mice (Araghi-Niknam, Miller, Sidwell, Fatemi, unpublished observations). Moreover, Reelin-expressing Cajal-Retzius neurons in the subpial layer are largely replaced by Reelin-expressing GABAergic interneurons that are dispersed throughout the mammalian neocortex (Rodriguez et al., 2000) and hippocampus (Fatemi et al., 2000a). Levels of the Reelin receptors ApoER2 and VLDLR and α3β1 integrin and the adaptor protein Dab-1, which are all essential to Reelin signaling, remain expressed in the adult brain (Weeber et al., 2002). Previous work by Rodriguez et al. (2000) showed an association between Reelin and its receptor α3β1 integrin with synaptic structures, raising the possibility of a potential role in neurotransmission. A recent report by Herz and colleagues (Weeber et al., 2002) shows that Reelin has a direct effect on the enhancement of long-term potentiation (LTP) in the hippocampus, which is abolished when hippocampus slice cultures are used from VLDLR and ApoER2 knockout mice lacking the receptors for Reelin. These investigators further report that Reelin and ApoER2 receptors cooperate to enhance hippocampal synaptic plasticity and learning (Weeber et al., 2002). Moreover, mice that lack the Reelin receptors ApoER2 or VLDLR have pronounced defects in memory formation and LTP (Weeber et al., 2002). Other behavioral and biochemical data also show that reductions in levels of Reelin in brain or blood, following postnatal hypoxia (Curristin et al., 2002), prenatal viral infection in midgestation (Fatemi et al., 1999; Shi et al., 2003), and in heterozygous Reeler mutants (Tueting et al., 1999) cause abnormalities in behavior, such as a decrease in prepulse inhibition, an increase in anxiety, and a decrease in memory formation. Additionally, mutations in the *RELN* gene have been associated with significant learning disability,

hypoplastic cerebellum (Hong et al., 2000), ataxia, and cognitive decline in human and mouse (Tueting et al., 1999). It is possible that variable deficits/polymorphisms in the *RELN* gene may be responsible for variable cognitive deficits in different mental disorders, including autism.

In conclusion, the above biochemical, neuroanatomic, and genetic data point to the involvement of Reelin in the pathophysiology of autism. Indeed, Reelin deficiency in the brain and blood of autistic individuals may be associated with brain structural abnormalities, as well as the profound communication and cognitive symptoms present in autism. Future, larger replications of these studies are warranted to determine the specificity of these alterations in the Reelin signaling system.

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26

Brain-Derived Neurotrophic Factor and Dopamine in Autism

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Research on the neurochemical changes that may underlie the developmental changes associated with autism has implicated a diverse range of neurotransmitter biomarkers. Some of these are discussed in detail in other chapters. Overall, many aspects of the disorder are consistent with an early role for neurotransmitter dysfunction in autism. This chapter focuses on recent neurochemical studies of two neurotransmitter systems that have a documented role in the etiology of autism: brain-derived neurotrophic factor (BDNF) and dopamine. In the past, the possible involvement of these systems was inferred from the results of numerous neuropharmacologic studies and from our understanding of their function in the normal brain. Recently, genetic analyses and studies using positron emission tomography (PET) have further confirmed the important role that the dysfunction of dopaminergic and BDNF neurons plays in the autistic brain. Although our understanding has progressed, problems related to consistent diagnosis and subtyping of the patients into comparable and homogeneous groups have confounded the interpretation of some of these studies. In addition, the interpretation of the findings discussed below may ultimately depend on a clear appreciation of whether these changes are simple epiphenomena unrelated to the actual causes of the disorder, end-stage changes that are not representative of the conditions that led to the disorder initially, or in fact somehow involved in the etiology of the disorder. The ultimate phenomenology of the disorder may depend on a more precise and broader understanding of the intricate interplay of dysfunction associated with many different neurotransmitter systems and not just the two considered here. The chapter begins by discussing the roles of each neurotransmitter in the normal brain and their interaction with one another; in this way, it will be possible to better appreciate the consequences of their dysfunction in autism.

Brain-Derived Neurotrophic Factor

BDNF is a polypeptide neurotrophin and a member of the same family of molecules that includes nerve growth factor (NGF), which is produced in the cell body and transported anterogradely to the nerve terminal, where it is stored in dense core vesicles and then released in an activity-dependent manner onto postsynaptic receptors (for a review, see Nawa and Takei, 2001). BDNF has been assigned a variety of roles in the brain, including the maintenance, growth, and differentiation of selected neuronal populations, particularly the forebrain cholinergic system (Nonner et al., 1996). Once released, BDNF acts by activating a tyrosine receptor kinase (trk), subtype A, that initiates a transautophosphorylation at specific tyrosine residues on the intracellular component of the receptor complex. These phosphorylated tyrosines function as docking sites for selected elements of intracellular signaling cascades that have numerous consequences; most important, the suppression of neuronal death (Ghosh et al., 1994; Kaplan and Miller, 2000). In a mouse model of Down syndrome, dying neurons fail to respond to BDNF signaling (Dorsey et al., 2002); a similar defect in the signaling efficiency of this and other neurotrophins might underlie aspects of the developmental problems associated with autism.

BDNF can also act on both the *N*-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) subtypes of glutamate receptors to influence their role in neuroplasticity (Yamada et al., 2002) and long-term enhancement (Sherwood and Lo, 1999), as well as the level of the neuronal form of nitric oxide synthase and calcium-binding proteins. Although BDNF can enhance NMDA-dependent neuronal responses to glutamate, it has been shown to protect against the excitotoxic apoptosis that can be initiated by glutamate (Glazner and Mattson, 2000). BDNF has a role in the enhancement of neural plasticity following exercise (Oliff et al., 1988; Mizuno et al., 2000). Its effects on learning might be related to its ability to enhance the release of glutamate and alter the function of glutamate receptors, as well as enhance the growth of dendrites in the hippocampus.

Aging is associated with elevated levels of BDNF in the hippocampus; this increase continues until age 24 months, when neural activity is considered to be moderately decreased (Katoh-Semba et al., 1998). In contrast to these changes seen in normal aging, the level of hippocampal BDNF decreases in patients with Alzheimer disease who have a significant memory dysfunction, as well as in aged senescence-accelerated mice (Phillips et al., 1991; Katoh-Semba et al., 1998). The elevation associated with normal aging might be required to maintain normal synaptic function (Katoh-Semba et al., 2001).

The expression of BDNF messenger RNA (mRNA) and BDNF protein in the hippocampus is apparently regulated by electrical and synaptic activity (McAllister et al., 1999). BDNF synthesis is stimulated in an activity-dependent manner. For example, in a recent study, the hyperexcitability induced by intraperitoneal injections of glutamate agonists or gamma-aminobutyric acid (GABA) antagonists lead to a marked and time-dependent increase in the level of BDNF mRNA in rats that developed convulsions. These drugs also increased levels of mature BDNF and its precursor in regions where levels of BDNF mRNA were enhanced, indicating the presence of newly synthesizing BDNF protein by the activation of the *BDNF* gene (Katoh-Semba et al., 2001).

Taken together, these recent findings suggest that in the autistic brain, the persistence of enhanced BDNF levels over many years (discussed in detail below), particularly during development, might cause a direct alteration of synaptic efficacy. In addition, this condition might trigger neuroplastic changes in synaptic transmission and induce an inappropriate reorganization. Furthermore, elevated levels of BDNF might enhance glutamate release in hippocampal neurons to such a degree that it could influence the establishment of hyperexcitability within the neural networks and predispose this brain region to epileptogenesis (Kokaia et al., 1995; Li et al., 1998).

Dopamine

Dopamine is used by several pathways within the central nervous system (CNS). The tuberohypophysial system (also known as the "tuberoinfundibular system") has cell bodies in the arcuate nucleus of the hypothalamus and periventricular nucleus. It projects axons to the median eminence and the intermediate and neural lobes of the pituitary and control the release of prolactin (Carlsson, 1993). Another dopamine projection system originates in the ventral mesencephalon. The mesocorticolimbic dopamine neuronal system is composed of cell bodies located in the ventral tegmental area (VTA) that send projections primarily to the frontal lobe structures, including the nucleus accumbens, olfactory tubercles, amygdala, septal area, and the prefrontal, cingulate, piriform, and entorhinal areas of the cortex. Although dopamine is the predominant neurotransmitter in species that evolved before molluscs, this neurotransmitter has a rather limited distribution in the human brain, compared to the other classical neurotransmitters.

These different dopaminergic systems subserve different functions. The nigrostriatal pathway consists of dopamine cell bodies in the substantia nigra that send axons to the striatum (caudate and putamen). Within these regions, dopamine has an important role in controlling movement. Decreased levels of dopamine in this pathway result in Parkinson disease. The symptoms of this dis-

order include tremors, spasticity, akinesia (difficulty in initiating or stopping movements). In contrast, increased levels of dopamine in the forebrain are associated with stereotypy in rats and schizophrenia in humans (Brunello et al., 1995). Forebrain dopamine also plays an important role in our emotional response to reward and the production of euphoria or the occurrence of depression (Fibiger, 1995). Virtually every known drug of abuse, or rewarding experience, stimulates directly or indirectly the forebrain dopamine neurons. This system is stimulated by the consumption of all known addicting substances, such as cocaine, amphetamine, heroin, or nicotine (Seiden et al., 1993). The complex interplay of the motor and reward functions of dopamine can be observed in rats given drugs that augment dopaminergic function and then placed in a running wheel; they begin to move forward as though they are running toward a rewarding stimulus. The range of complex behavioral functions outlined above that have been attributed to dopamine has led to a consideration of its role in the symptoms of autism and influence on theory of mind tasks (Dennis et al., 1999).

INTERACTIONS BETWEEN DOPAMINE AND BDNF

Dopamine and BDNF may be released from the same neurons in the striatum. The dopaminergic system may influence the developmental regulation of *BDNF* gene expression in the striatum (Kuppers and Beyer, 2001). In contrast, BDNF induces long-term changes in brain function by influencing the responsiveness of its target neurons to dopamine (Guillin et al., 2001). Furthermore, BDNF is capable of inducing the expression of the tyrosine hydroxylase enzyme in human and rat fetal tissues; these cells can then produce and release dopamine (Theofilopoulos et al., 2001). Mice lacking an allele for BDNF have elevated levels of dopamine in their striatum, possibly due to decreased release of this transmitter in response to neuronal excitation (Dluzen et al., 2002). Estrogen may also exert a stimulatory effect on the differentiation of the dopaminergic system via its ability to regulate the expression of BDNF mRNA and protein levels during development of the female nervous system (Ivanova et al., 2001). Estrogen alpha receptors and BDNF are colocalized to the same pyramidal neurons in the CA3 and CA1 regions of the developing hippocampus. Estrogen has recently been demonstrated to interact directly with BDNF to influence hippocampal physiology during development (Solum and Handa, 2002). The estrogen dependency of these mechanisms may underlie aspects of the gender-related severity of autism (i.e., although the demonstrated male:female ratio in probands with higher IQ is 4:1, autistic females tend to be more severely affected than males). Female autistic patients usually have a much lower IQ

than do the males and a higher incidence of major neurologic abnormalities (Tsai et al., 1981).

DOPAMINE AND AUTISM

Impaired function of the dopaminergic system in autistic brains would be consistent with our current understanding of the role of forebrain dopamine neurons in normal cognitive function. For example, dopamine plays a role in controlling movement, attention, perception, and a variety of social behaviors; each of these functions is impaired in autistic patients. However, in the previous edition of this volume, Anderson (1994) concluded that no significant differences existed between control and autistic children with regard to dopaminergic function. Forebrain dopaminergic activity has often been inferred by monitoring changes in urinary levels of the major dopamine metabolite, homovanillic acid (HVA). This measure might not be reliable, because only a minor percentage of the urinary HVA is actually derived from the CNS. In spite of some recent progress, the controversial role of dopamine has not been completely resolved.

Recently, a study of 14 autistic children (both boys and girls) used PET scanning of fluorine-18-labeled fluorodopa to measure presynaptic dopaminergic activity in the caudate nucleus, putamen, midbrain at the level of the substantia nigra, prefrontal, and occipital cortex (Ernst and Zametkin, 1997). The uptake of the label was reduced significantly only in the anterior medial prefrontal cortex in the autistic children, and this change was unrelated to the individual's IQ scores.

The dysfunction of the dopaminergic system in the autistic brain is also supported by genetic evidence for polymorphic AC repeat in the gene for dopamine beta-hydroxylase (Robinson et al., 2001). Dopamine beta-hydroxylase is the enzyme responsible for the conversion of dopamine to norepinephrine. The imbalance of dopaminergic and noradrenergic function in utereo might underlie aspects of the symptomatology after birth. The A1 allele of the Taq I polymorphism of the dopamine type D2 receptor gene has been shown to occur in a very high percentage of alcoholics; the prevalence of the A1 allele was demonstrated to be significantly increased in autistic patients (Comings et al., 1991). Although not conclusive, taken together, these findings are consistent with the hypothesis that altered dopaminergic function plays a critical role in the etiology of autism, and its dysfunction clearly underlies aspects of the symptoms associated with this disorder.

BRAIN-DERIVED NEUROTROPHIC FACTOR AND AUTISM

Very little information currently exists regarding the precise role of BDNF in the autistic brain. A recent pair of studies (Nelson et al., 2001; Perry et al., 2001) con-

cluded that the significant elevation in the level of BDNF in blood and brain—specifically, the region of the nucleus basalis of Meynert—is an intrinsic component of the disease rather than evidence of some unknown compensatory mechanism that selectively involves this neuropeptide. Figure 26.1 shows that the increased level of BDNF in the basal forebrain region of the autistic brain is specific and not a general property of the neurotrophins (e.g., NGF). Alternatively, the up-regulation of BDNF levels might be a consequence of the excessive physical activity (Oliff et al., 1998) that is typically exhibited by these children. Genetically engineered mice that overexpress BDNF demonstrate impaired performance in a learning and memory task and show increased susceptibility to seizures (Croll et al., 1999), symptoms that are consistent with those seen in children with autism.

Pharmacotherapy of Autism

A better understanding of the role of these two systems in autism may lead to an effective pharmacotherapy. One problem has been that autism is an insidious developmental disorder that is clinically heterogeneous. In general, the potential therapeutic benefit of therapies directed at dopaminergic dysfunction has been confirmed indirectly by recognition that drugs that antagonize dopaminergic receptors produce consistent clinical benefit (Minderaa et al., 1989). The classic neuroleptic drugs can reduce levels of some of the most problematic behaviors associated with autism, including stereotypy and self-injurious behavior (Patzer and Volkmar, 1999). In support of this interpretation, similar symp-

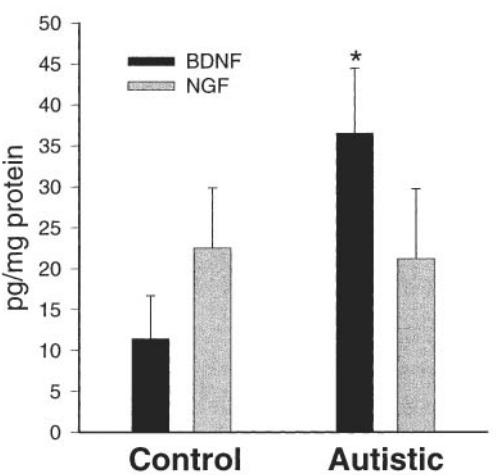

FIGURE 26.1. Endogenous levels of BDNF and nerve growth factor (NGF), expressed as picograms per milligram of protein, in the basal forebrain of autistic and control subjects. The mean ages were 17.77 and 13.62 years for the control and autistic groups, respectively. Values shown are the group mean ± standard deviation. The asterisk indicates $p = 0.02$ versus controls. *Source:* Data from Perry et al. (2001).

toms have been induced in patients and animals by drugs that enhance the release of dopamine (e.g., stimulants). Finally, a recent double-blind, placebo-controlled study found that risperidone significantly reduced the incidence of repetitive behavior, aggression, anxiety, depression, and irritability (McDougle et al., 1998). No therapies have yet been proposed or designed to influence BDNF function in the autistic brain during development.

Conclusion

The dysregulation of the function of an important growth factor, such as BDNF, coupled with the dysfunction of a neurotransmitter, such as dopamine, that is critical for so many cognitive and motor skills, may underlie the expression of specific symptoms associated with autism. A recent report suggested that changes in these and other biomarkers might also be associated with a heterogeneous group of developmental disorders (Nelson et al., 2001). Recent investigations into the genetic abnormalities underlying autism have identified multiple loci on a majority of the human chromosomes (Risch et al., 1999). Although the changes in function of these neurotransmitters might be simply epiphenomena that are indirect consequences of the genetic abnormalities, a better understanding of these abnormalities, and the behavioral changes that are a consequence to them, might assist in the development of better diagnostic tools for the identification of infants at risk of the disorder.

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27

The Immune System

Andrew W. Zimmerman, M.D.

For more than 30 years, investigators have sought to define immune functions in children with autism and their families. In 1971, Chess observed that some children with congenital rubella syndrome also had autism, and Money and colleagues (1971) described a family with autism who also had several autoimmune disorders. Controversies persist regarding viral etiologies (including persistence of measles virus) and autoimmune theories (e.g., autoantibodies affecting the brain). Abnormalities and atypical patterns occur in nearly all types of immune functions in autism. Most have been described in small groups of subjects with various clinical definitions for autism. Little data directly link immune findings in peripheral blood to abnormalities in the brain in autism, and thus their relevance remains uncertain.

Several theories of immune pathogenesis in autism have led to experimental immune treatments. Despite promising anecdotal reports of success, there have been no published placebo-controlled trials of immune therapies. This chapter reviews immune findings in autism, along with immune theories and possible treatments, and discusses areas in which further research is needed.

Immune Findings

Several types of immune functions in autism have been reviewed (van Gent et al., 1997; Burger and Warren, 1998; Zimmerman, 1999; Korvatska et al., 2002; Krause et al., 2002). Those presented in this chapter are humoral (B-cell), cellular (T-cell), and innate (cytokines, natural killer [NK] cells, complement) immune functions, and human leukocyte antigen (HLA) studies. Most of these studies have been exploratory and have included a small series of subjects ($N = 10–50$) with subjective diagnostic criteria for autism, and measure individual immune factors at single time points and ages. We do not know if changes occur with growth of a

child with autism, and whether reported immune "deficiencies" reflect "dysmaturation" of the immune system that may normalize as the child grows.

HUMORAL AND CELLULAR IMMUNITY

Stubbs (1976; Stubbs and Crawford, 1977) found that nearly half in a series of 15 autistic children studied (ages 3–10 years) had no detectable rubella antibodies, despite having received the vaccine, whereas all controls had normal titers. All of these children developed titers following revaccination, and their responses did not segregate according to the severity of their diagnoses. Decreased lymphocyte transformation in response to stimulation with phytohemagglutinin (PHA) was also noted but not to pokeweed mitogen (PWM). It was suggested that a relative state of anergy might exist in autism that could be developmental. In clinical practice, children with autism seem to have an increased frequency of childhood infection (especially otitis media), although Comi et al. (1999a) were not able to confirm this in a survey using parental recall. Furthermore, there have been no reports of serious immune deficiency states in autistic children, although the immunoglobulin IgA and other isolated IgG subclass deficiencies occur in 30–60 percent of children, ages 3–12 years (Zimmerman et al., 1995; Gupta et al., 1996). Conversely, Ferrari et al. (1988) found increased IgG and responses to stimulation of B-cells with PHA.

These early findings raised the possibility that prenatal viral infections might damage the immature immune system and induce tolerance to a virus (Stubbs and Crawford, 1977). Studies of cellular immune functions by Warren and colleagues (1986, 1990b) have shown deficient numbers of T-cells and a selective decrease in CD4 + T-helper (Th)/inducer cells, as well as depressed proliferative responses by T-cells to mitogens. Singh (1988) found abnormal mitogenic responses (both high and low), and Yonk (1990) noted a specific deficiency of suppressor-inducer T-cells (CD4+CD45AR+) in children with autism. Plioplys et al. (1994) and Denney et al. (1996) found increased DR+ T-cell activation (without the expected interleukin [IL]-2 receptor activation), and Gupta et al. (1998) reported altered ratios between Th1 and Th2 cell subsets. These findings suggest a possible autoimmune pathogenesis in autism, as similar changes are known to occur in rheumatoid arthritis, lupus, and multiple sclerosis (Warren et al., 1990b).

In the only report of cell-mediated activity against a specific central nervous system (CNS) antigen in autism, Weizman (1982) reported inhibition of macrophage migration to myelin basic protein (MBP) in cells from 13 of 17 children with autism but in none of the controls. Singh and colleagues (1993, 1998) also reported the presence of serum antibodies to purified MBP in 58 percent of

autistic children studied, compared to 9 percent of controls. There is no pathologic evidence of demyelination in autism, and recent findings using quantitative magnetic resonance imaging (MRI) show *increased* myelin content with macrocephaly (Herbert, 2001).

Self-reactive antibodies might result from specific viral or bacterial infections in a predisposed immune system and then later cross-react with specific cells or receptors in the brain through the process of molecular mimicry (Albert and Inman, 1999). They might also arise de novo through an autoimmune process, such as fetal-maternal incompatibility in the womb. This process could result in either (or both) maternal or fetal immune reactivity that would affect fetal brain development, and would continue to exert its effects after birth. For example, a maternal antibody response to fetal brain antigens early in gestation might alter brain maturation at critical stages and have more serious consequences postnatally. Maternal antibodies were found in six of 11 mothers who reacted with their autistic child's lymphocytes (Warren et al., 1990a). In children with autism, several antibodies have been detected in sera that react to (nonautistic) brain tissue. These include antibodies to the frontal cortex (Todd, 1988; Plioplys, 1989), cerebellum (Zimmerman et al., 1993), and cerebral endothelium (Connolly et al., 1999). Because antibodies in these studies were not tested against autistic brain tissue (which may express different antigens than controls do), their relevance to autoimmune reactions in autism is unknown.

Recently, antibodies in serum from mothers of children with dyslexia and autism, when injected into pregnant mice, bound to Purkinje cells and other large neurons, and affected behavior and motor coordination in the offspring (Dalton et al., 2003). Maternal autoantibodies may therefore be more relevant to prenatal brain development than to the postnatal effects of antibodies in autistic children or children with other developmental disorders (Vincent et al., 2003).

Circulating antibodies that react to purified CNS proteins (including neuronal-axon filament protein [NAFP] and MBP) are detectable in sera from children with autism, in association with antibodies to human herpes virus-6 and measles-IgG, as well as cross-reactive peptides from milk and common pathogens (Singh et al., 1998; Vojdani et al., 2002). Antibodies to serotonin and its receptors have been described, but do not explain changes in serotonin levels in autism (Yuwiler et al., 1992; Cook et al., 1993).

Children with autism may be prone to develop a wide range of self-reactive antibodies. Similar antibodies are also found, to lesser degrees, in typical children but do not normally cross an intact blood brain barrier. They could, however, be produced endogenously in the CNS (Cserr and Knopf, 1997; Hickey et al., 1997). Such antibodies or immune cell antigens might provide useful clinical markers, if they could be correlated with clinical and familial phenotypes. A

good example of this concept is the B lymphocyte antigen D8/17, found in 78 percent of autistic patients but in only 21 percent of controls, and associated with high scores for compulsive behaviors (Hollander et al., 1999). Another example is the presence of nonspecific antibodies found in Tourette syndrome (Loiselle et al., 2003). The D8/17 antigen has also been associated with rheumatic fever, and correlates with the repetitive behaviors observed in Sydenham chorea, as well as in Tourette syndrome.

INNATE IMMUNE FUNCTIONS

Intracellular signaling by cytokines in the immune system mirrors the actions of neurotransmitters in the CNS. Cytokines can initiate and modulate signaling pathways, act within the CNS, and affect brain development and behavior (Malek-Ahmadi, 2001). Singh et al. (1991) found increased levels of T8 antigen (sT8) and IL-2 antigen (sIL-2), but not soluble IL-2 receptor or soluble IL-1 in the sera of 23 children with autism. Further study showed increased plasma levels of IL-12 and interferon-gamma (IFN-γ) in 20 children with autism (mean age, 10.7 years) compared to controls, but no differences in levels of IFN-γ, IL-6, tumor necrosis factor (TNF)-α, or soluble intercellular adhesion molecule-1 (sICAM-1) (Singh, 1996). Analyzing cytokines in lymphocyte subsets, Gupta et al. (1998) found decreased proportions of Th-1 and increased Th-2 cells in 20 young autistic children (4–8 years old), compared to controls. T-cell subsets containing increased IL-4 were consistent with increased serum IgE in autism (Gupta et al., 1996). This was the first study of intracellular cytokines, and it showed a shift from Th-1 to Th-2 subsets, consistent with earlier observations of depressed cell-mediated immunity (Stubbs and Crawford, 1977; Warren et al., 1986).

Two investigators approached the cellular expression of cytokines in vitro by incubating lymphocytes from children with autism and controls. Croonenberghs (2002) found increased production of IFN-γ, IL-1RA, IL-6, and TNF-α in 13 teenagers after incubation of unstimulated whole blood supernatants for 48 hours. In 71 children with autism, Jyonouchi and colleagues (2001) stimulated innate immune responses in peripheral blood mononuclear cells using lipopolysaccharide and found increased expression of TNF-α, IL-1β, and IL-6, compared to controls. Elevated cytokine responses were also produced by exposure of the cells to common dietary proteins (Jyonouchi et al., 2002).

Cytokines can act bidirectionally between the immune and central nervous systems. Therefore, such atypical increased peripheral cellular immune responses in autism might parallel endogenous production of cytokines by

immune competent cells in the CNS, such as microglia and astrocytes (Korvatska et al., 2002). This may be especially important for TNF-α production by activated microglia, specialized immune cells in the CNS. Once they are activated by infection, proinflammatory cytokines, antibodies, neuronal degeneration, or apoptosis, the microglia are capable of initiating a cascade of excitotoxicity, mediated by glutamate and its receptors, which could become self-perpetuating (Zimmerman and Myers, 1999). For example, TNF-α normally enhances activity-dependent synaptic efficacy by increasing surface expression of AMPA (a type of glutamate) receptors in glial cells and may thereby modulate synaptic plasticity (Beattie et al., 2002). Conversely, excessive glutamate-mediated excitatory activity may lead to the overproduction of TNF-α and thus, more glutamate. This would lead to a continuing feed-forward cycle of glutamate-mediated excitotoxicity that may play a role in autistic regression (Figure 27.1).

However notable these immune findings in peripheral blood, there have been few neuropathologic studies of microglia (or other immune activity) in autistic brain tissue that would confirm or deny the presence of immune activation in the CNS. One study of microglial activation products (quinolinic acid, neopterin, and biopterin) in cerebrospinal fluid from 12 children with autism (ages 3–12 years) showed no differences from age-matched controls (Comi et al., 1999b). Recently, microglial activation (as well as increased staining of astrocytes) was observed in postmortem autistic brain tissues from 12 subjects (Vargas et al., 2003).

NK cells provide nonspecific (innate) immune surveillance before and after birth. Warren et al. (1987, 1990b) found reduced NK cell activity (but normal numbers of NK cells) in 12 of 31 subjects with autism, compared to controls. This decreased activity could increase the risk of acute or chronic viral infection (or viral persistence) in the fetus or neonate.

Complement is also important for innate immune recognition and the marking of pathogens for clearance by immune cells. Warren et al. (1991, 1994) described a deficiency specifically for the C4B type of complement protein in 49 percent of 45 autistic subjects, compared to 79 controls. Decreased levels were also found in the parents and siblings. Increased frequencies of the null allele for C4B (no protein produced) occurred in subjects with autism, as well as their mothers (Warren et al., 1991). Despite low complement levels, no clinical signs of immune deficiencies or symptoms of autism (e.g., in the mothers) have been associated with either the null allele or low circulating levels of C4B, presumably because of redundancy within alternate pathways. The gene locus for C4B on chromosome 6p is of interest. It lies within the coding region for the major histocompatibility complex (MHC), which includes HLA and TNF-α.

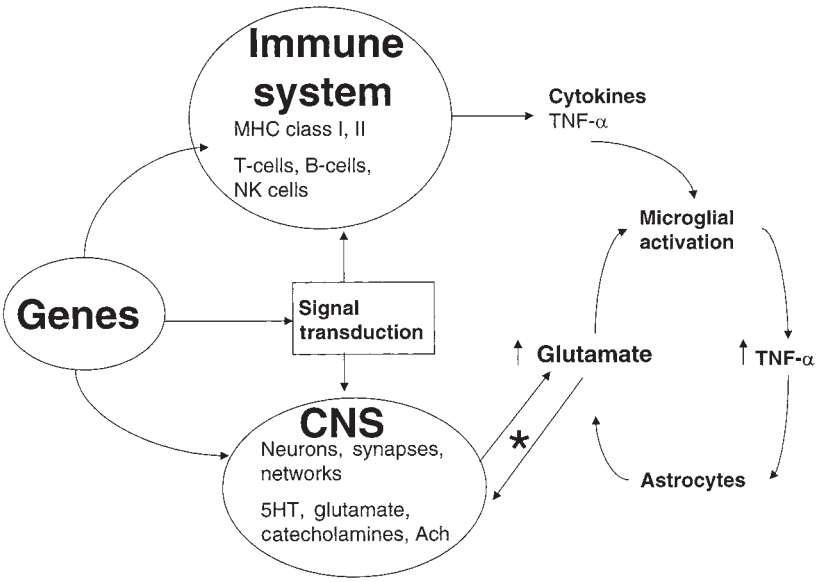

FIGURE 27.1. The immune system and central nervous system (CNS) respond to genetic controls and interact dynamically through signal transduction. In autism, cytokines (such as tumor necrosis factor [TNF]-α) may induce microglia and astrocytes in the CNS to respond with additional TNF-α and glutamate in a feed-forward loop of excitotoxicity. This may contribute to autistic regression (*). Ach, acetylcholine; 5HT, 5-hydroxytryptamine (serotonin); MHC, major histocompatibility complex; NH, natural killer.

Major Histocompatibility Complex and Human Leukocyte Antigens

Genetic determinants of the immune system reside within the MHC on chromosome 6 and code for HLA (for a review, see Burger and Warren, 1998). More than 100 genes code for highly polymorphic HLA molecules, which have multiple forms or alleles. HLA class I molecules are present on the surfaces of nearly all cells and enable cells to be recognized as part of the "self." If mismatched, they lead to rejection of tissue transplants. HLA class II molecules are expressed on cell surfaces, including circulating white blood cells, resident macrophages, and microglia in the brain. They bind peptides from pathogens and "nonself" molecules and display them on the surface of antigen-presenting cells for recognition by T-cells (Janeway and Medzhitov, 2002). Certain HLA antigens are also associated with autoimmune disorders, such as rheumatoid arthritis and lupus (Klein and Sato, 2000).

Studies of HLA types in children with autism and their parents suggest possible relationships to autoimmune disorders or fetomaternal incompatibility.

Specific HLA types were found more commonly in some groups of children with autism or their parents, compared to control groups or population norms (Stubbs and Magenis, 1980). However, findings have varied among groups under study, possibly due to small sample sizes, methods for typing, heterogeneity of clinical characteristics of autism, and differences in populations. The most consistent findings have been increased frequencies of HLA-DR4 (class II) and related DRβ1 alleles in autism, which were also found to be associated with a null allele of C4B in 49 percent of subjects with autism, compared to 16 percent of controls (Warren et al., 1996a). An increased frequency of the combined extended ancestral haplotype (B44-SC30-DR4) was found in children and mothers, more frequently than in fathers (Warren et al., 1992; Daniels et al., 1995). Further subtyping of HLA-DRβ1 alleles showed several alleles to occur more commonly in autistic subjects than in controls (Warren et al., 1996b).

The close proximity of gene loci for HLA-DR, C4B, and TNF-α in the MHC on chromosome 6p suggests that these genes or their products may interact in some way, affecting immune functions in autism. This suggestion was supported by Warren et al. (1995), with their finding of activated DR+ T-cells, along with decreased levels of C4B protein, in the peripheral circulation in nine of 20 (45%) autistic subjects compared to 11 percent of controls.

HLA types may be associated with increased susceptibility to autoimmune disorders. In a survey of 61 families, Comi et al. (1999a) found increased rates of autoimmune disorders (especially rheumatoid arthritis, which is frequently associated with DR4) in mothers and first-degree relatives of children with autism, although not in the children themselves. Notably, the risk of autism increased (odds ratio: from 1.9 to 5.5) as the number of family members with an autoimmune disorder increased from one to three. The increased incidence of autoimmune disorders in the mothers also may have been underestimated, due to their ages at the time of the study, as women may develop autoimmune disorders later in life.

HLA patterns of parental inheritance have been inconsistent. In a study of 103 families, Torres et al. (2002) found HLA-DR4 in 50 probands with autism, and in 13 fathers and 23 mothers, using samples from Oregon and Utah. DR4 in this study was more likely to be inherited from the father than the mother. HLA findings may differ with geographical or population groups, and chance associations with apparent statistical validity may occur with small sample sizes (or clinical subtypes of autism). Our own studies show that maternal DR4 is increased in a random sample of 30 families with idiopathic autism, but not in 30 multiplex families with autism spectrum disorder (Zimmerman et al., 2001). Rogers et al. (1999) could not confirm linkage to the HLA region in 90 multiplex sibships with autism, although the marker loci they used might have missed critical regions in the MHC. If autism turns out to have an autoimmune component in even a small

percentage of families, HLA genes might confer susceptibility by a novel mechanism. For instance, recent studies in type 1 (autoimmune) diabetes using transgenic mice show how HLA class II genes provide susceptibility or protection from disease (Undlien and Thorsby, 2001).

Theoretical Approaches

The weight of evidence and possible analogies to the CNS strongly imply that there might be links between brain function and the immune system in autism. However, there are no convincing clues that delineate which immune findings may reflect causes of autism and which may be its effects.

INFECTIONS

Warren et al. (1994) suggested that immune dysregulation in autism may predispose some children to infections or autoimmunity. This idea has been proposed as a possible link to vaccine effects in autism through a hypothetical response to immunizations (Halsey and Hyman, 2001). Although it is theoretically possible that immune stimulation from vaccines could have greater (or lesser) cerebral effects in children with autism compared to typically developing peers, examination of currently available data does not support this concept (DeStefano, 2002).

Several direct infections of the brain, both prenatal and postnatal, have been associated with autism, including rubella (Chess, 1971, 1977), cytomegalovirus (Stubbs et al., 1984), and herpes encephalitis (Gillberg, 1991). A report of measles virus persistence in bowel biopsies in autism, using a sensitive reverse transcriptase–polymerase chain reaction assay (Uhlmann et al., 2002) has methodologic problems and has not been confirmed or studied in autistic postmortem brain tissue (Offitt, 2002).

Patterson (2002) emphasized the importance of maternal infections during pregnancy for their effects on fetal brain development. This was based in part on epidemiologic evidence of an association of maternal influenza during gestation with an increased risk of schizophrenia, an effect that has been replicated in a mouse model (Shi et al., 2001). Descriptions of polymorphisms in the TNF-α and IL-1 genes in schizophrenia suggest, by analogy, that cytokines themselves might function abnormally in autism (Katila et al., 1999; Boin et al., 2001).

A NEUROIMMUNOLOGIC DISORDER?

The concept that prenatal immune and hormonal interactions between mother and fetus may affect brain development as a neuroimmunologic disorder has

endured since the work of Geschwind and Galaburda (1985). It was hypothesized that this relationship might lead to later presentations of neurodevelopmental disorders during childhood (e.g., autism) or adolescence (e.g., schizophrenia) (see Pearlson, 2000).

To date, only the finding of microglial activation in autistic brain tissue suggests that this pleomorphic syndrome might have neuroimmunologic aspects, although it would not be a typical neuroimmunologic disorder. There are few signs of inflammation in postmortem brains (Bauman and Kemper, 1994; Bailey et al., 1998), and circulating autoantibodies or activated T-cells recognizing CNS antigens, described in autism, have not been shown to be specifically reactive to autistic brain tissue. Established criteria for autoimmune disorders require evidence of immune involvement from clinical signs and disease course, disease replication through transfer of pathogenic antibodies or T-cells, and reproduction of the autoimmune disease in experimental animals (Rose and Bona, 1993). These criteria have been typically fulfilled by multiple sclerosis, myasthenia gravis, and Guillain-Barré syndrome. Unlike autism, these disorders can have their onset at different ages, have typical clinical signs and courses, and show laboratory markers of the disease processes (Pender and McCombe, 1995). Autism follows a time-dependent clinical course in the first 3 years of life, and most children improve in different degrees over time.

GENES

Large genome screens have not found signals near the MHC locus on chromosome 6p. This implies that if genetic immune determinants are involved in the pathogenesis of autism, they are not acting primarily, but may have secondary or *epigenetic* effects through gestational or postnatal autoimmune processes. *Epistatic,* or cooperative gene interactions, may also occur with genes lying in the MHC. Genetic loci for autoimmune and inflammatory disorders also cluster with those for autism and Tourette syndrome, and suggest a genetic relationship based on immune dysregulation (Becker et al., 2003).

Single genes may simultaneously affect immune and CNS functions, through polymorphisms that reduce gene expression. For example, adenosine deaminase (ADA), an enzyme found on the cell surface of lymphocytes and neurons, is important for immune activity, purine metabolism, and peptidase activity. Of the two known alleles of the gene for ADA, *ADA2* is associated with lower catalytic activity. Recently, individuals carrying one copy of *ADA2* were found more frequently (18%–31%) among autistic patients, compared to 8–14 percent of controls (Persico et al., 2000; Bottini et al., 2001).

The appearance, timing, and levels of gene expression are critical for dynamic

changes taking place during brain development, for the formation and migration of neurons and glia, for subsequent synaptogenesis, and for the development of interactive networks (Zimmerman and Gordon, 2000). The major excitatory neurotransmitter, glutamate, and its receptors are essential for long-term potentiation and the establishment of stable synapses (Johnston et al., 2001). Recently, increased expression of two glutamate-related genes (*EAAT1* and *AMPA1*) was found in autistic postmortem brain tissue (Purcell et al., 2001). Glutamate-related excitotoxicity might contribute to the early and age-dependent regression noted in some autistic children between 18 and 24 months of age (Gordon, 1992; Zimmerman and Myers, 1999), when glutamate activity and cerebral glucose utilization peak (Chugani et al., 1987; Kornhuber et al., 1989). Glutamate leads to synaptic pruning, necessary for eliminating excessive connections that have developed up to that time.

MAJOR HISTOCOMPATIBILITY COMPLEX AND MICROGLIA

Shatz and colleagues demonstrated in mice that MHC class I gene expression is critical for normal development of the visual system (Corriveau et al., 1998). One usually associates MHC molecules with alerting the immune system to kill infected cells and rejecting foreign tissue transplants. Like MHC class II molecules, class I molecules are normally present in the brain at low levels. Class I MHC gene expression increases during critical periods of brain development. Like glutamate, these proteins promote pruning and elimination of unnecessary synapses by enhancing long-term potentiation (Boulanger et al., 2001). Further studies may show that MHC class I molecules are also present in widespread areas of the human brain at critical periods, and that timing or levels of their gene expression may be delayed or deficient in autism and other neurodevelopmental disorders.

Microglia may be important links between the CNS and immune system in autism. The microglia are resident CNS macrophages and are present early in the fetal brain. They increase in numbers during gestation (Hickey et al., 1992; Wierzba-Bobrowicz et al., 1998), and are important for developmental plasticity (Santambrogio et al., 2001). Postnatally, with signals from astrocytes and cytokines, microglia become "activated" (Rezaie et al., 2002), express MHC class II molecules, and present class II–restricted antigens to T-cells. When highly activated, microglia produce cytokines, such as IL-1β, IL-6, TNF-α, complement, superoxide radicals, and neurotoxic factors. In turn, TNF-α induces astrocytes to release glutamate, which can then stimulate its own receptors on microglia to release additional TNF-α (see Figure 27.1), amplifying excitatory mechanisms (Bezzi et al., 2001).

Activated microglia may constitute one common pathway by which several types of insults to the developing CNS (e.g., metabolic disorders, infections, drug effects, immune disorders) could produce autism. Microglial activation has been observed in schizophrenia and Alzheimer disease (Bayer et al., 1999; Radewicz et al., 2000), and has led to clinical trials of anti-inflammatory drugs (in t' Veld et al., 2001; Mueller et al., 2002).

Immune Treatments

Several types of immune treatment have been proposed or attempted in autism. Although there have been anecdotal reports of improvements, no placebo-controlled trials of these therapies have been reported (Zimmerman, 2000). The most dramatic anecdotal reports have come from the use of corticosteroids, although there have been no laboratory studies of immune functions to determine if changes occur with treatment (Mott et al., 1996; Chez et al., 1998). Intravenous immunoglobulin replacement has shown promise in a few children in two small series, suggesting that there might be a subgroup that can respond to this therapy (Gupta et al., 1996; Plioplys, 1998). However, a third study showed no changes following such treatment (DelGiudice-Asch et al., 1999). If direct studies of immune activity in the brain support neuroimmune effects in autism, controlled anti-inflammatory drug trials may become feasible.

Conclusion

Despite multiple studies of immune differences in autism, this area of research has been hampered by a lack of unifying hypotheses, consistent and comprehensive data with clinical definitions and correlations, and continuous follow-up of subjects and immune functions over time. Many important questions remain, as well as the need for testable hypotheses, before controlled therapeutic trials become compelling priorities.

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Epilogue

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Autism remains an enigmatic, clinically defined syndrome for which we have no clear understanding of the etiology or the pathogenesis of the structural and behavioral manifestations. Although a number of etiologic hypotheses have been proposed, a genetic basis for the disorder remains the leading theory. There has been a good deal of interest in the study of specific syndromes with known genetic causes, such as fragile X syndrome and tuberous sclerosis, in which a percentage of affected individuals exhibit autistic features, with the hope that understanding these disorders could provide significant etiologic leads. Although the possibility of a single gene disorder has not been completely ruled out, most investigators are now focusing on the hypothesis that autism may be the result of the expression of multiple abnormal genes acting in concert, perhaps in relation to some additional, as yet unspecified environmental factor. This polygenetic theory is intriguing, inasmuch as it could account for the varied clinical manifestations seen in affected individuals and for the presence of autistic-like traits among close relatives. A number of candidate genes have been identified, including the genes for gamma-aminobutyric acid (GABA) on chromosome 15, the serotonin transporter gene (SLC6A) on chromosome 7, and the gene for adenosine deaminase (ADA), an enzyme that plays a role in the regulation of adenosine, on chromosome 20. At this point in time, the most frequently reported duplication has been identified in the chromosome 15q1–13 region, which contains the genes for at least 15 GABA receptor subunits. Whether these (and probably others still to be identified) genetic abnormalities are inherent to the affected infant or whether they are related to genetic abnormalities in the mother, which then create an unfavorable intrauterine environment for the fetus, remain open questions.

Neuroanatomic changes have been observed in many areas of the brain, with evidence for several different pathologic processes. The timing of these processes appears to extend from the early stages of embryological development into

adulthood. This diversity of timing raises two hypotheses. It is possible, for example, that abnormalities occurring very early in gestation may have a significant impact on specific cell lines and/or neuronal circuitry, which would then affect subsequent brain development. Alternatively, it may be that the varied morphologic changes reflect the influence of multiple abnormal genes, with or without adverse environmental factors, that affect the brain during different times throughout the life of the patient. Clinical studies of thalidomide exposure during pregnancy have shown an increased risk for autism if the mother received this substance during the first 20–24 days postconception, suggesting a possible critical period of brain development. Neuropathologic studies on the brains from several autistic subjects exposed to thalidomide have shown malformations of the inferior olive of the brainstem. Agenesis of the facial nerve nucleus and superior olive has been reported in a single case of autism, implicating involvement during the fifth or sixth week, a slightly later period of gestation.

Still later in development is the critical period for the development of malformations of the cerebral cortex. Although such malformations have been rarely and inconsistently reported, their presence implicates a pathologic process that dates to the prenatal period. In our own material, we have noted consistent neuroanatomic changes in the limbic forebrain and in the cerebellum and related brainstem nuclei, suggesting that these regions may be important to the core pathology of autism. In the limbic forebrain, the predominant finding is an increased cell packing density and a decreased neuronal size in the hippocampus, amygdala, and entorhinal cortex, and in the cerebellum, a decreased number of Purkinje cells and changes in their related cerebellar nuclei and inferior olive. Purkinje cells are normally generated during the sixth and seventh weeks of gestation. Assuming that these cells are properly generated at that time, we have hypothesized that, given their known prenatal connection with the olivary climbing fibers, the reduced numbers of Purkinje cells noted in the autistic brain must have occurred before the 28th fetal week.

In postnatal development, one of the most striking findings is an abnormal increase in brain weight and volume and in head circumference. Although not present at birth, these features appear to be evident by at least 2 years of age. At later ages, the rate of brain growth in the autistic brain has been reported to be less than that in typically developing children, and enlarged head size is no longer evident. Although there have been variable reports of enlargement of gray matter volume, the weight of the existing evidence leans heavily toward an increase in the volume of the white matter in the forebrain and cerebellum. Preliminary neurochemical studies of the corpus callosum, obtained from adult autistic brains, suggests an immaturity of myelin development, with selective alterations in glycolipids and phospholipids.

Further postnatal changes include the presence of abnormally enlarged neurons of the cholinergic nucleus of the diagonal band of Broca (NDB), and in the cerebellar nuclei and inferior olive observed in all of our autopsied autistic brains less than 13 years of age. In contrast, all of the autistic brains older than 21 years show reduced neuronal size and number in these same structures, these changes apparently occurring during adolescence. How or whether these neuronal alterations impact on the clinical features in autism is unknown. The NDB is intimately related to the hippocampus, and the cerebellar nuclei and inferior olive are intimately related to the cerebellar cortex, and it is conceivable that the changes may be associated with the primary abnormalities in their target areas. Alternatively, these changes may be related to abnormalities in the myelinated fibers that interconnect the nuclei with their target regions.

Neurochemical studies have implicated abnormalities in neurotransmitters, growth factors, and peptides in autism. Most frequently mentioned are the neuropeptide BDNF (brain-derived neurotrophic factor) and the neurotransmitter GABA. BDNF is thought to have a role in the maintenance, growth, and differentiation of selective neuronal populations, particularly the forebrain cholinergic system. BDNF has been noted to be increased in the autistic brain in the basal forebrain and cerebral cortex, and in blood samples obtained from newborn infants who later developed autism. Abnormalities in GABA are of equal interest. Multiple GABA subunits have been identified on chromosome 15. In addition, the GABAergic system has been associated with seizure disorders, and it is known that approximately one-third of autistic individuals have seizures at some time during their lives. It is also known that GABA plays an important role in early brain development, and abnormalities of this neurotransmitter may be, at least in part, responsible for the immature appearance of the neurons in the limbic forebrain. GABA receptors have been found to be decreased in the hippocampal formation, a finding supported by observations using positron emission tomography (PET) in a subset of autistic individuals.

Although increased levels of serotonin have frequently been reported in peripheral blood platelets obtained from autistic individuals, a robust abnormality of serotonin in the central nervous system has yet to be identified. However, a delay in the postnatal time table of serotonin metabolism in the autistic brain relative to controls has been identified with PET, and similar studies have shown asymmetries in serotonin synthesis in the circuitry connecting the frontal cortex, thalamus, and cerebellum in a small series of autistic boys. So far, less attention has been paid to other neurotransmitters, especially cholinergic, noradrenergic, and dopaminergic systems, but some early data are beginning to emerge. For example, a loss of nicotinic and muscarinic receptors have been reported in the cerebral and cerebellar cortex, a finding that would support the

neuropathologic changes noted in the heavily cholinergic nucleus of the diagonal band of Broca in the septum.

Recently, Reelin, a glycoprotein with important functions in brain development and as well as in adult life, has been reported to be abnormally expressed in the brain and blood of autistic individuals. In the brain, decreased levels have been found in the cerebellum and in the blood in a significant number of affected individuals and in their immediate family members. Similar findings have also been noted in other neuropsychiatric disorders, suggesting that this perturbation may represent a vulnerability factor for these disorders.

In spite of continued efforts to implicate the immune system in autism, there remains no convincing evidence that it plays a significant role. Although no naturally occurring or genetically derived animal models are available for autism research, at least two pathologic models have provided insights into the relationships between abnormal brain structures and clinical features. These models are of particular interest, as the experimenter can control the time of the insult and the location of the lesion and follow the effect of the artificially created insult on later morphologic, neurochemical, and behavioral development. Borna virus disease in neonatal rats has provided one such model and has a number of similarities to autism in brain pathology, neurochemistry, and behavior. Anatomic similarities include the loss of Purkinje cells and involvement of the hippocampus. However, there are also significant morphologic differences. The Purkinje cell loss in Borna virus disease is postnatal rather than prenatal, and abnormalities in the hippocampus are associated with the loss of granule cells, rather than with the curtailment of neuronal maturation seen in human autism. Borna virus has also been associated with a loss of large neurons in the cerebral cortex, findings not observed in the autistic brain. Nonetheless, there may be useful lessons to be learned from these affected animals, most especially, a better understanding of the relationships between specifically located early brain lesions and their resultant behavioral patterns with maturity.

Monkey models of autism are of particular interest because of their close phylogenetic relation to man and their well-developed social behaviors. Recent primate studies have shown that early, combined damage to the orbitofrontal cortex and amygdala produce persistent changes in emotional responses and social behavior. The observations suggest that these structures are important components for the development of such behaviors and may be critical to our understanding of some of the clinical characteristics of autism.

The clinical features of autism continue to be a fertile and intensive area of research. It is critical that the basic science investigator understand the subtleties of receptive and expressive language and nonverbal communication, as well as multiple aspects of atypical information processing that are characteristic of this

disorder. Such information adds critically needed observations for the laboratory scientist, raising important investigative questions and providing a clinical profile against which basic science findings can and should be held up to scrutiny. Basic science often yields multiple observations, but it is important that these findings "make sense," given the clinical presentation of the patient.

Over the past 10 years, we have increasingly come to appreciate that autistic individuals can and often do have associated medical and psychiatric disorders that need to be investigated and addressed. There is now an increased awareness that high levels of serotonin are present in the gastrointestinal (GI) track and that the vagus nerve sends extensive information from the GI tract to the brain. What specific roles these abnormalities may play in the symptomatology of autism remains an important area of active research. Although there is no one medication available that can reliably and positively impact on some of the atypical clinical features of autism, it is clear that a number of medications have been utilized successfully to manage some of the negative behavioral symptoms that impede developmental progress in autistic patients. There continues to be an active search for better, more precise pharmacologic interventions, which, with improved understanding of the underlying neurochemical substrate of this disorder, will no doubt become available in the future.

The topics included in this book represent a sampling of some of the very important autism research now under way worldwide. Autism is no longer considered a rare disorder, and funding for much needed basic science and clinical investigations has increased substantially since the publication of the first edition of this book. It continues to be our hope that by bringing multiple perspectives together in a single volume, important research questions will be stimulated, thereby leading to a better clarification and more in-depth understanding of the neurobiologic basis of autism, and ultimately to more precise and effective modes of early identification and treatment.

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